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Kinetics and Mechanism of the Association of the Bacteriophage T4 Gene *32* (Helix Destabilizing) Protein with Single-stranded Nucleic Acids

Evidence for Protein Translocation

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We have investigated the association kinetics of the co-operatively binding T4-coded gene *32* (helix destabilizing) protein with a variety of single-stranded homopolynucleotides (both RNA and DNA). Stopped-flow mixing experiments were performed by monitoring the partial quenching of the intrinsic tryptophan fluorescence of the protein upon binding to the nucleic acid under conditions where the nucleic acid concentration is in great excess over the protein concentration. Investigations of the association rate (and rate constants) as a function of solution variables has demonstrated quite different behavior at the extremes of "low" and "high" salt concentration. Under low salt (high binding constant) conditions the non-co-operative association is rate-limiting and we measure a bimolecular rate constant of 3×10^6 to 4×10^6 M^{-1} (nucleotide) s^{-1} (0·1 M-NaCl, 25·0°C). However, at higher salt concentrations (lower binding constant) a pre-equilibrium involving non-co-operatively bound protein is established, followed by the rate-limiting formation of co-operatively bound protein clusters.

Based on these observations we have proposed a mechanism for the formation of co-operatively bound T4 gene *32* protein clusters, under conditions of low binding density, which consists of three steps: (1) pre-equilibrium formation of non-co-operatively bound protein (nucleation); followed by (2) association of free protein to the singly contiguous sites established in the nucleation step, hence forming the first co-operative interactions (growth step); and (3) a redistribution of the growing protein clusters to form the final equilibrium distribution. From comparisons of our experimental values of the forward rate constant for the second step (growth of clusters) with theoretical estimates based on the work of Berg & Blomberg (1976,1978) we infer that the T4 gene *32* protein is able to translocate along single-

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stranded polynucleotides. The implications of these results for the *in vivo* action of the T4 gene *32* protein are discussed.

1. Introduction

The gene *32* protein, encoded by bacteriophage T4, is a helix destabilizing protein that is necessary for DNA replication, recombination and repair in T4-infected *Escherichia coli* (Epstein *et al.*, 1963; Tomizawa *et al.*, 1966; Alberts *et al.*, 1968; Alberts & Frey, 1970; Alberts & Sternglanz, 1977). The role of gene *32* protein during DNA replication has been the most intensely studied of all its functions (Alberts *et al.*, 1980; Liu *et al.*, 1978). Its presumed role, as a result of its high affinity for single-stranded DNA, is to bind to the transient single-stranded regions of DNA in order to both protect the DNA from nucleases and to hold the DNA in a conformation facilitating replication by DNA polymerase (Alberts & Sternglanz, 1977; Liu *et al.*, 1978; Alberts *et al.*, 1980).

The equilibrium binding properties of the gene *32* protein to single-stranded oligonucleotides and polynucleotides (both RNA and DNA) have been extensively studied by von Hippel and co-workers (Jensen *et al.*, 1976; Kelly & von Hippel, 1976; Kelly *et al.*, 1976; Kowalczykowski *et al.*, 1981*b*; Newport *et al.*, 1981; Lonberg *et al.*, 1981). These properties, as well as those of the other helix destabilizing proteins have recently been reviewed (Williams & Konigsberg, 1981; Kowalczykowski *et al.*, 1981*a*; Coleman & Oakley, 1980). The G32P† binds co-operatively to single-stranded DNA and RNA with a nearest neighbor co-operativity parameter, ω (McGhee & von Hippel, 1974; Schellman, 1974) of $\sim 10^3$ to 10^4, which is fairly independent of base composition and salt concentration (Jensen *et al.*, 1976; Kowalczykowski *et al.*, 1981*b*; Newport *et al.*, 1981). Although G32P binds non-specifically to single-stranded nucleic acids, in the sense that it will bind anywhere along the lattice regardless of base composition, Newport *et al.* (1981) have determined that G32P does exhibit a preference for binding to the homodeoxyribopolynucleotide over the corresponding homoribopolynucleotide. In addition, G32P displays a base specificity in its binding to homopolynucleotides (Bobst & Pan, 1975; Newport *et al.*, 1981). The equilibrium binding of G32P to single-stranded polynucleotides is extremely sensitive to the salt concentration in solution (Kowalczykowski *et al.*, 1981*b*) as is the case for most DNA binding proteins (Record *et al.*, 1976,1978). The dependence of $K\omega$ on [NaCl] was determined to be $(\partial \log K\omega/\partial \log [\mathrm{NaCl}]) = -6 \pm 1$ (Kowalczykowski *et al.*, 1981*b*; Newport *et al.*, 1981). The rate constants for the interaction also reflect this salt sensitivity and we have exploited this in our studies of the mechanism of interaction presented here.

The kinetics of the interaction of G32P with single-stranded polynucleotides are of interest for several reasons.

(1) The *in vivo* processes of replication, recombination and repair do not occur under equilibrium conditions. Hence we need to know what steps in these processes are rate-limiting under *in vivo* conditions, so that we can understand how these

† Abbreviation used G32P, protein product of gene *32*.

processes are controlled. In the case of DNA replication, which occurs at a rate of $\sim 10^3$ nucleotides s^{-1} (McCarthy *et al.*, 1976), knowledge of the rates with which the various replication proteins can interact with DNA is obviously important since the interaction of any one protein can be rate-limiting for the entire process. Kinetic information may also allow us to determine the mechanism by which G32P moves with the replication fork.

(2) Jensen *et al.* (1976) found that although G32P is a helix destabilizing protein, by virtue of its higher affinity for single-stranded over duplex DNA, it is unable to achieve equilibrium and melt native duplex DNA (although it can melt poly[d(A·T)]) under conditions where the G32P–single-stranded DNA complex is thermodynamically favored. The kinetic studies reported here and in subsequent papers will form a basis for understanding this kinetic block to melting native DNA. Curiously, G32P seems to be the only helix destabilizing protein that has this kinetic block.

(3) The G32P–single-stranded polynucleotide system presents an opportunity to conveniently study the association kinetics of a *non-specific* protein–nucleic acid interaction, since the intrinsic tryptophan fluorescence of G32P is partially quenched upon binding to any region of the polynucleotide (Kelly & von Hippel, 1976; Kelly *et al.*, 1976). All previous protein–nucleic acid association kinetic studies have focused on *specific* binding as in the case of the *E. coli lac* repressor–operator association (Riggs *et al.*, 1970; Barkley, 1981; Winter *et al.*, 1981), or the *E. coli* RNA polymerase–promoter association (Hinkle & Chamberlin, 1972*a*,*b*; Maquat & Reznikoff, 1978; Giacomoni, 1979; McClure, 1980; Belintsev *et al.*, 1980).

In this paper we describe the association kinetics of G32P to single-stranded homopolynucleotides as a function of solution conditions and polynucleotide type. In addition to obtaining kinetic information which may be relevant to the role of G32P *in vivo*, this work also provides a systematic study of a non-specific protein–nucleic acid association. There have been a number of kinetic studies on the non-specific association of various ligands to polynucleotides and DNA. Pörschke (1976,1978,1979*a*,*b*,1980) has investigated the kinetics of binding of Mg^{2+} and various positively charged oligopeptides to single-stranded oligonucleotides and polynucleotides. The relaxation kinetics of DNA complexed with intercalating dyes, drugs and steroids have also been studied (Müller & Crothers, 1968; Müller *et al.*, 1973; Li & Crothers, 1969; Bresloff & Crothers, 1975; Dattagupta *et al.*, 1978; Capelle *et al.*, 1979; Wakelin & Waring, 1980). However, this is the first investigation of the association kinetics of a non-specific protein–nucleic acid association.

The attempt to explain the seemingly anomalously large association rate constant for *lac* repressor binding to its operator which is contained in a large piece of non-specific DNA (Riggs *et al.*, 1970) has resulted in a substantial body of theoretical work (Richter & Eigen, 1974; Berg & Blomberg, 1976,1977,1978; Berg, 1978; Lohman *et al.*, 1978; Schranner & Richter, 1978; Berg *et al.*, 1981). Both the experimental and theoretical work on the *lac* repressor–operator association have revealed several interesting features. Most notably, *lac* repressor appears to "slide" (i.e. translocate without dissociating) along duplex DNA (Richter & Eigen, 1974; Berg & Blomberg, 1976,1978; Barkley 1981; Winter *et al.*, 1981). This "sliding"

facilitates the fast sampling of nucleotide sequences by repressor until the operator is contacted, and also accelerates the association to the operator through the use of an extended target (i.e. the non-specific DNA). Another mechanism that has been proposed to facilitate the sampling of nucleotide sequences is "direct transfer" (von Hippel *et al.*, 1975; Bresloff & Crothers, 1975). This process requires two DNA binding sites on the ligand and the formation of an intermediate wherein the protein is simultaneously bound to two sites on the DNA. Subsequent dissociation from one DNA site enables the ligand to be transferred ($\sim 50\%$ of the time) to the other site which is on the same DNA molecule. This mechanism is thought to play a role in the redistribution of DNA-bound ethidium (Bresloff & Crothers, 1975) and some bisintercalators (Capelle *et al.*, 1979; Wakelin & Waring, 1980).

One would like to know what aspects of the various proposed mechanisms mentioned above are generally relevant and available to all proteins (e.g. direct transfer, sliding, hopping). Kinetic data on a number of DNA binding proteins are needed to answer these general questions and the results presented here for G32P will hopefully provide some general insights into the kinetic pathways of other protein–nucleic acid systems, as well as its own.

2. Materials and Methods

All chemicals used in this study were reagent grade. Buffered solutions were made with twice distilled water and were: buffer T (10 mM-Tris (hydroxymethyl) aminomethane, 0·1 mM-Na_3EDTA, pH 8·3) and buffer H (10 mM-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 0·1 mM-Na_3EDTA, pH 7·5). In initial experiments, 0·75 mM-β-mercaptoethanol was used in the buffers, but this was later eliminated with no apparent effect on the gene *32* protein. Therefore the majority of the experiments described here were done in the absence of β-mercaptoethanol. The salt concentrations of the buffers were adjusted by addition of NaCl or NaF to the indicated value. The pH of the buffers did not vary by more than $\pm 0{\cdot}1$ pH unit over the salt concentration range studied.

We have not observed any effect of pH on the association kinetics with the 2 buffer conditions used in our experiments. However, we have observed a small, but complex pH dependence of the dissociation rate of co-operatively bound G32P–single-stranded nucleic acid complexes (Lohman, unpublished observations).

(a) *Polynucleotides*

Homopolynucleotides (except poly(rϵA)) were purchased from Miles Biochemicals and Collaborative Research, and were used without further purification. The average sedimentation coefficient of these samples varied from 4·5 S for poly(rA) to 7·5 S for poly(dT). Poly(1,N^6-ethenoadenylic acid) (poly(rϵA)) was purchased from P-L Biochemicals. Concentrations were determined using the extinction coefficients listed by Kowalczykowski *et al.* (1981*b*).

(b) *T4 gene* 32 *protein*

Two preparations of T4 gene *32* protein were used in this study. Both were prepared using the procedure of Alberts & Frey (1970) as modified by Jensen *et al.* (1976). One preparation is described by Kowalczykowski *et al.* (1981*b*) wherein *E. coli* B was infected with a triple T4 mutant (T4 33^-, 55^-, 61^-) in Super Luria broth. The other procedure used a T4 double mutant (T4 33^-, 55^-) to infect *E. coli* BE (grown to 5×10^8 cells/ml) in M9S media. (T.M.L. is grateful to Drs D. Rabussay and E. P. Geiduscheck for supplying the T4 mutant and

E. coli hosts.) The two G32P preparations behave identically, both electrophoretically and in the kinetics experiments reported here. The G32P is $> 95\%$ pure as judged by polyacrylamide gel electrophoresis and its concentration was determined spectrophotometrically using an extinction coefficient of $3{\cdot}7 \times 10^4$ M^{-1} cm^{-1} (Jensen *et al.*, 1976). The G32P was stored in 0·1 M-NaCl, 20 mM-Tris (pH 8·1), 1 mM-EDTA, 0·5 mM-dithiothreitol, 50% glycerol at -20°C.

In the calculations presented in this paper, a binding site size of $n = 7$ nucleotides and a molecular weight of 35×10^3 was used for gene *32* protein. This differs by only 4% from the molecular weight of 33,500 determined from the amino acid sequence (Williams *et al.*, 1980).

(c) *Stopped-flow kinetics*

Rapid mixing experiments were performed using a modified Durrum-Gibson stopped-flow spectrophotometer equipped with fluorescence detection. The mixing dead time of the instrument is 2·5 ms. A Hamamatsu R 1104 photomultiplier tube was used. The light source was a 150 W xenon lamp (Osram) and the original monochromator was replaced by a Bausch and Lomb monochromator. The kinetics experiments monitored changes in the gene *32* protein fluorescence using an excitation wavelength of 290 nm. For experiments with polynucleotides other than poly(rεA), we used a glass microscope slide as a cutoff filter placed directly in front of the photomultiplier tube to eliminate wavelengths < 310 nm; for G32P, $\lambda_{em,max} = 347$ nm (Kelly & von Hippel, 1976). Poly(rεA) is also fluorescent ($\lambda_{ex,max} = 309$ nm, $\lambda_{em,max} = 406$ nm). Therefore, in experiments involving poly(rεA) which monitored the G32P fluorescence, an interference filter centered at 343 nm was used in the emission pathway to eliminate this background signal. The time-course of the experiments was displayed on a storage oscilloscope. The stopped-flow is also interfaced to a Varian 620i computer and the output from each experiment was stored on magnetic tape for processing and analysis. All experiments were conducted at $25{\cdot}0 \pm 0{\cdot}2$ deg. C unless noted otherwise.

The kinetic traces were analyzed from the magnetic tape using the Varian 620i. The time-courses were fit to either a single exponential decay, $F(t) - F(\infty) = \Delta F = Ae^{-t/\tau}$, or a sum of 2 exponential decays $\Delta F = Ae^{-t/\tau_f} + Be^{-t/\tau_s}$, where $F(\infty)$ is the fluorescence signal at final equilibrium. The parameters τ_f, τ_s, A, B and $F(\infty)$ were obtained using a least squares fitting procedure (CFIT) which uses various subroutines obtained from the Harwell Subroutine Library. (We thank Professor C. Klopfenstein and G. Remington for the writing and implementation of this program.)

The use of the intrinsic G32P fluorescence to monitor the kinetics enabled us to work at relatively low protein concentrations. The range of concentrations was 0·1 to 0·4 μM although we typically worked at $\sim 0{\cdot}2$ μM. At these concentrations $> 99\%$ of the G32P exists as monomer while free in solution (Carrol *et al.*, 1972,1975).

3. Results

In our interpretations of the kinetic data, we have assumed that the observed quenching of the intrinsic protein fluorescence, upon mixing G32P with single-stranded nucleic acids, is due to the formation of protein–nucleic acid contacts rather than protein–protein contacts. From this assumption it follows that the quantum yield of G32P is identical when it is bound to the nucleic acid in any of its three binding modes (i.e. isolated, singly or doubly contiguous binding). The experimental evidence that supports these assumptions is briefly summarized here.

(1) The extent of G32P fluorescence quenching varies with the particular nucleic acid lattice (Kelly & von Hippel, 1976; Kowalczykowski *et al.*, 1981*b*) indicating that protein–nucleic acid interactions are at least partly responsible for the ΔF.

(2) The main evidence is based on experiments that compare the fluorescence quenching of G32P, G32P*I and G32P*III upon binding nucleic acids; G32P*I and

G32P*III are formed by tryptic digestion of G32P (Moise & Hosoda, 1976). G32P*I is missing 48 amino acids from the C terminus of G32P, but still binds co-operatively to single-stranded nucleic acids with a value of $\omega = 10^3$, while G32P*III is missing both the C-terminal fragment and 21 amino acids from the N terminus of G32P and no longer binds co-operatively, i.e. $\omega = 1$ (Lonberg *et al.*, 1981). Equilibrium binding studies of G32P, G32P*I and G32P*III to poly(dT) at low salt indicate that the intrinsic tryptophan fluorescence of all three proteins is quenched to the same extent ($\sim 52\%$) (Kowalczykowski *et al.*, 1981*b*; Lonberg *et al.*, 1981). Since G32P*III does not form co-operative protein–protein interactions, this suggests that the ΔF observed for G32P is due only to protein–nucleic acid interactions. Identical extents of quenching are also observed for all three proteins upon binding to the oligonucleotide $d(pT)_8$, where none of the proteins can form protein–protein interactions.

(3) In addition to monitoring the time-course of association *via* the quenching of the intrinsic G32P fluorescence, we have also followed the time-course by monitoring the enhancement of poly(rϵA) fluorescence upon binding of G32P. This fluorescence enhancement is the result of unstacking of the bases in poly(rϵA) (Steiner *et al.*, 1973; Kowalczykowski *et al.*, 1981*b*). Under identical conditions, the time-course observed by monitoring the G32P is exactly equivalent to the time-course obtained by monitoring the poly(rϵA) (data not shown). This supports the idea that the quenching of the G32P fluorescence is the result of binding to the nucleic acid lattice rather than protein–protein interactions, since it is quite unlikely that optical signals from the protein and nucleic acid would be identical if the protein signal resulted partly from co-operative interactions.

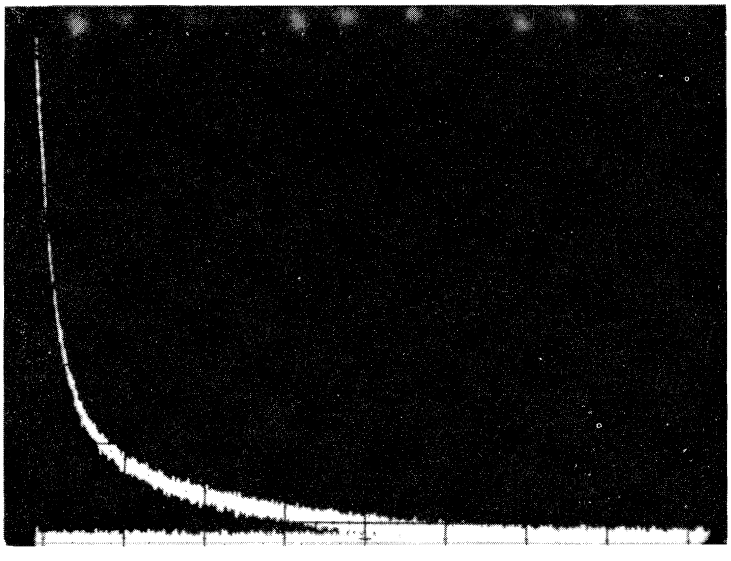

FIG. 1. Oscilloscope trace of the T4 gene *32* protein–poly(rA) association kinetics, monitoring the quenching of the intrinsic tryptophan fluorescence of the protein (0·10 M-NaCl, buffer T (pH 8·3), 25·0°C). [G32P] = 0·125 μM; [poly(rA)] = 13·0 μM (nucleotides); final fractional saturation of the poly(rA) is 6·3% (λ_{ex} = 290 nm; time scale = 2 s/cm; vertical scale = 100 mV/cm).

The time-course of a typical association reaction with the nucleic acid concentration (hereafter designated [NA]) in excess over the G32P concentration is shown in Figure 1. The decrease in the G32P fluorescence, upon binding to the single-stranded nucleic acid has been monitored. The majority ($> 90\%$) of the time-courses that we have observed can be described very well by a sum of two exponential decays. The faster of the two exponential decays is designated by a time constant τ_f, and the slower by a time constant τ_s. Typically, a series of experiments was performed at a constant [G32P] while varying the [NA]. Under the conditions of our experiments the amplitude of the slow phase (τ_s) was always less than 25% of the total observed amplitude. However, the amplitude of τ_s decreases with increasing [NA], eventually disappearing at very high [NA] where a single exponential decay, with a time constant corresponding to τ_f, is observed.

In the majority of the experiments reported here, the amplitude of τ_s was only ≤ 10 to 15% of the total amplitude. As a result it was difficult to obtain very accurate estimates of τ_s and its dependence on solution variables. Therefore, we have not attempted a quantitative analysis of the slow phase, but only report its general characteristics. τ_s^{-1} was found to be independent of [NA] and temperature at a constant salt concentration. At 0·10 M-NaCl, $\tau_s^{-1} = 0{\cdot}7 \pm 0{\cdot}6\ s^{-1}$, but decreases with increasing [NaCl]. The dependence of τ_s^{-1} on the type of homopolynucleotide is also negligible, within experimental error. Further discussion of this slow phase along with our interpretation of τ_s is given in the Discussion.

The majority of this paper focuses on the fast relaxation time, τ_f, and its dependences on solution variables and concentration of protein and polynucleotide. In general, the behavior of the fast relaxation time is determined by the salt concentration of the solution. The dependence of τ_f on solution variables and nucleic acid concentration is qualitatively different depending on whether the reaction is carried out in "strong binding" (generally low salt) or "weak binding" (generally high salt) conditions. (These conditions are defined by the observed kinetic behavior, which is described below.) We first present the data for the association kinetics under strong binding conditions, to be followed by the weak binding association kinetics.

(a) *Association behavior in the strong binding region*

(i) *Nucleic acid concentration dependence*

In Figure 2 we show the dependence of τ_f^{-1} on [NA] (in units of M (nucleotides)) at constant [G32P], ([NA] $\gg$ [G32P]) for the association of G32P with poly(dT) and poly(rεA) in buffer T (0·10 M-NaCl). As is apparent in Figure 2, τ_f^{-1} is directly proportional to [NA] under these pseudo-first-order conditions. The association behavior with poly(rI) (data not shown) is identical to that of poly(dT) and poly(rεA) at 0·1 M-NaCl. That is to say, no specificity is observed in the association rate even though the equilibrium constant, $K\omega$, for co-operative binding of G32P to these three polynucleotides ranges over two to three orders of magnitude under these conditions (Newport *et al.*, 1981). The value of τ_f is independent of [G32P] as long as the nucleotide concentration is in excess of the [G32P] (we have gone as high as [G32P]/[NA] = 0·07, i.e. a final fractional saturation of the nucleic acid equal to 0·49 without any change in τ_f).

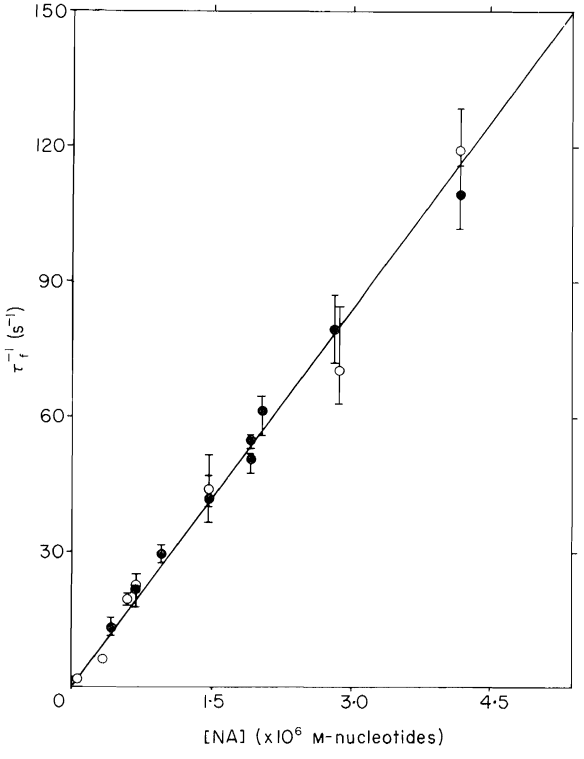

FIG. 2. Nucleic acid concentration dependence of τ_f^{-1} (at constant [G32P] = 0·21 μM) for the T4 G32P association with (●) poly(dT) and (○) poly(reA) (0·10 M-NaCl, buffer T(pH 8·3), 25·0°C).

The observed behavior of τ_f^{-1} as a function of [NA] can be described by a simple bimolecular association mechanism (P ≡ G32P; D ≡ nucleic acid):

$$P + D \underset{k_d}{\overset{k_a}{\rightleftarrows}} PD. \tag{1}$$

The relaxation time for this mechanism, when the [NA] is in excess over the protein concentration is (Eigen & de Maeyer, 1963):

$$\tau^{-1} = k_a[D] + k_d. \tag{2}$$

The apparent association rate constant obtained from the slope of Figure 2 has the value $k_a(\text{app}) = 2{\cdot}8 \pm 0{\cdot}4 \times 10^6$ M^{-1} (nucleotide) s^{-1} (0·10 M-NaCl, 25·0°C). (Consideration of the appropriate units for k_a will be presented in the Discussion.) As judged by the value of the intercept in Figure 2, $k_d(\text{app})$ is very small and indistinguishable from zero. (The reference to an apparent association rate constant, $k_a(\text{app})$, throughout the text signifies a rate constant which has not yet been related to molecular rate constants: see equation (11).) Since $k_d(\text{app}) = 0$, we see that τ_f^{-1} is simply a pseudo-first-order rate constant, under strong binding conditions; i.e. $\tau_f^{-1} = k_a[D]$ from equation (2).

In the following sections we present measurements of k_a(app) as a function of salt concentration, temperature, viscosity and nucleic acid length. This is done since k_a(app) should change in characteristic ways as a function of these variables if the rate-limiting step is the bimolecular association of G32P with the nucleic acid, under strong binding conditions.

(ii) [*NaCl*] *dependence of the association rate and lack of nucleic acid specificity under strong binding conditions*

In Figure 3 we show the effect of [NaCl] on τ_f^{-1} in the strong binding region for the association of G32P with poly(rεA) in buffer H. At all three [NaCl] (20 mM, 52 mM, 100 mM), τ_f^{-1} is directly proportional to [poly(rεA)]; however the apparent rate constant, k_a(app), (given by the slope) decreases with decreasing [NaCl]. At 52 mM-NaCl, $k_a(\text{app}) = 1{\cdot}3 \pm 0{\cdot}2 \times 10^6\ \text{M}^{-1}$ (nucleotide) s^{-1} and at 20 mM-NaCl, $k_a(\text{app}) = 2{\cdot}7 \pm 0{\cdot}1 \times 10^5\ \text{M}^{-1}$ (nucleotide) s^{-1}. In the case of the association at 20 mM-NaCl, the linear dependence of τ_f^{-1} on [poly(rεA)] persists to high nucleic acid concentrations ($\sim 10^{-4}$ M-nucleotides).

Kowalczykowski *et al.* (1981*b*) have shown that $K\omega$ increases upon substituting fluoride for chloride in the range 0·3 to 0·7 M-NaCl (NaF). Under strong binding conditions (0·1 M-NaCl (NaF), poly(rεA)) we find that k_a(app) is independent of the anion used (data not shown).

Experiments similar to those in Figure 3 were also with poly(dT) and poly(rI) as a function of [NaCl]. In all cases, the rate of association decreases with

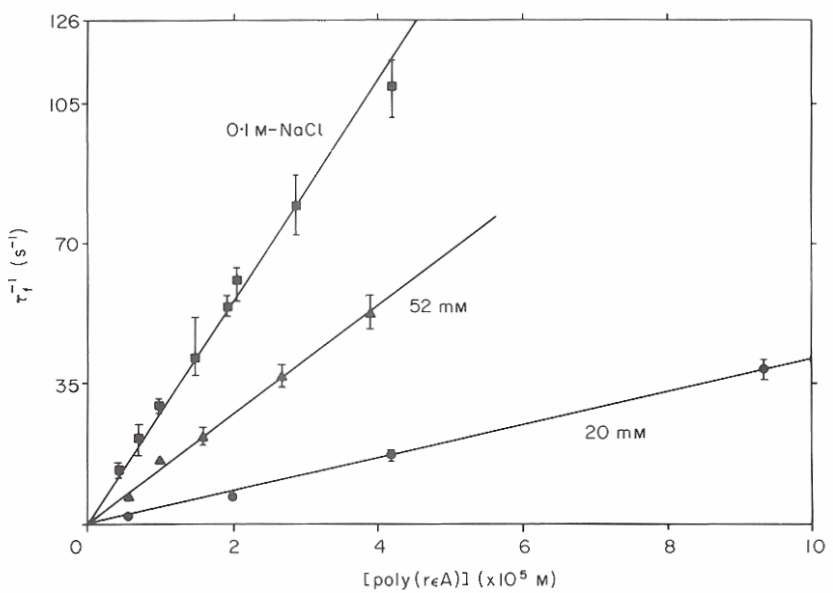

FIG. 3. T4 G32P–poly(rεA) association as a function of [NaCl] under strong binding conditions. (τ_f^{-1} *versus* [NA] at constant [G32P] = 0·21 μM). 100 mM-NaCl, $k_a(\text{app}) = 2{\cdot}8 \times 10^6\ \text{M}^{-1}$ (nucleotide) s^{-1}; 52 mM-NaCl, $k_a(\text{app}) = 1{\cdot}3 \times 10^6\ \text{M}^{-1}$ (nucleotide) s^{-1}; 20 mM-NaCl, $k_a(\text{app}) = 2{\cdot}7 \times 10^5\ \text{M}^{-1}$ (nucleotide) s^{-1}.

decreasing [NaCl]. The apparent association rate constants in the strong binding region are summarized in Figure 4 (open symbols), where they are displayed on a log–log scale. Although the data are scattered, k_a(app) is essentially independent of homopolynucleotude (i.e. there is no specificity; see also Fig. 2). (The scatter seems to be due to a difference in average molecular weight among the three polynucleotides; see section on length dependence.) The dependence of k_a(app) on [NaCl] in the region 20 mM $\leq$ [NaCl] $\leq$ 100 mM in Figure 4 is:

$$\log k_a(\text{app}) = 1{\cdot}5(\pm 0{\cdot}3) \log [\text{NaCl}] + 7{\cdot}95. \quad (3)$$

At [NaCl] > 0·10 M, only the association of G32P with poly(dT) remains in the strong binding limit. In Figure 4 we observe that strong binding conditions persist to 0·3 M-NaCl for poly(dT). In addition, k_a(app) $\simeq 4 \times 10^6$ M^{-1} (nucleotide) s^{-1} at 0·20 M and 0·25 M-NaCl, which is slightly larger than the value measured at 0·10 M-NaCl.

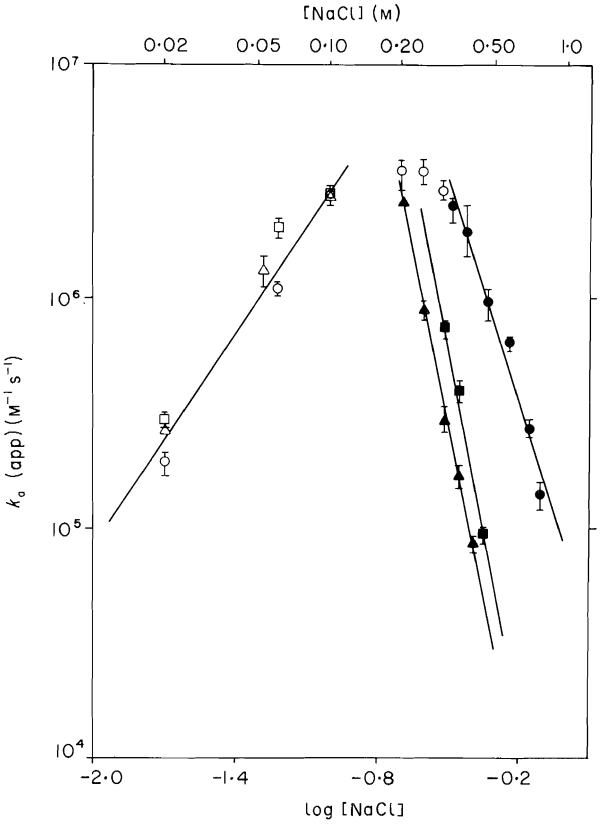

FIG. 4. [NaCl] dependence of k_a(app) (log–log plot) for poly(dT) (○, ●); poly(rεA) (△, ▲) and poly(rI) (□, ■). Open symbols correspond to strong binding association behavior. Filled symbols correspond to weak binding association behavior. The weak binding data have been obtained at 3 different [G32P] and hence the specificity shown here is not quantitative although the qualitative specificity is correct (see Fig. 9). (●) [G32P] = 0·125 μM; (■) [G32P] = 0·40 μM; (▲) [G32P] = 0·21 μM.

At this point, we note that under the pH conditions of these experiments, the G32P has a net negative charge (pI = 5; Alberts, 1974) although its nucleic acid binding site is presumably positively charged. Hence, the increase in k_a(app) with increasing [NaCl] is expected if the rate-limiting step in this strong binding region is the approach of the negatively charged G32P to the negatively charged nucleic acid. The increase in [NaCl] will screen the charges of the two molecules and facilitate their mutual approach. Therefore the observed [NaCl] dependence of k_a(app) indicates that we may be measuring the bimolecular rate of non-co-operative association for this system.

(iii) *Temperature dependence*

We have measured the temperature dependence of k_a(app) for the G32P–poly(dT) association under the strong binding conditions in buffer H (0·1 M-NaCl). The results are given in Table 1, from which we calculate an activation energy of

TABLE 1

Temperature dependence of k_a*(app)*† *under strong binding conditions*

Polynucleotide	k_a(app) = k_1($\times 10^{-6}$ M^{-1} (nucleotides) s^{-1})	Temperature (deg. C)
Poly(dT)	2·8±0·2	20·5
	3·0±0·3	25·0
	3·3±0·1	30·8
Poly(rεA)	2·9±0·2	25·0
	3·6±0·2	30·8

† Buffer H (0·10 M-NaCl, pH 7·6±0·1).
E_a = 5±2 kcal/mol.

5±2 kcal/mole. This very small, but positive value for the activation energy is also consistent with a reaction which is limited by diffusion.

(iv) *The association rate is dependent upon the length of the nucleic acid lattice*

We have compared the association rate of G32P with two poly(rI) samples of widely different molecular weights in buffer H, 0·1 M-NaCl. The fractionated poly(rI) samples had $\langle s_{20,w} \rangle$ of 2·5 S and 12 S corresponding to average lengths of 50±20 nucleotides and 1500±200 nucleotides, respectively, based on the studies of Eisenberg & Felsenfeld (1967). For the 12 S poly(rI) sample we measure k_a(app) = $2{\cdot}4 \times 10^6$ M^{-1} (nucleotide) s^{-1} which is the same as that obtained for poly(dT) and poly(rεA) in Figure 2. However, the 2·5 S poly(rI) sample has a much higher value of $6{\cdot}0 \times 10^6$ M^{-1} (nucleotide) s^{-1}.

(v) *Viscosity dependence of the association*

If the rate-limiting step, under strong binding conditions, is the bimolecular association, then the effect of the solution viscosity on k_a(app) should be

substantial. We first used glycerol to change the solution viscosity; however the endpoint in the fluorescence change indicated that the equilibrium binding constant was substantially lowered in the presence of $> 10\%$ glycerol. Sucrose solutions did not change the endpoint for the G32P–poly(dT) association in buffer T (0·1 M-NaCl, 25·0°C). An equilibrium measurement at high salt, indicated that $K\omega$ decreases by no more than a factor of two in the presence of 20% sucrose. We therefore used sucrose to change the solution viscosity (η).

Values of k_a(app) were determined for the G32P–poly(dT) association in buffer T (0·1 M-NaCl) in solutions with sucrose concentrations varying between 0 and 20%. We observed that k_a(app) is a linear function of T/η and the linear least squares fit to the data is given by:

$$k_a(\text{app}) = 87(\pm 12)T/\eta + 2(\pm 2) \times 10^5,$$

where (T/η) and k_a(app) have units of (°K poise^{-1}) and M^{-1} (nucleotide) s^{-1}, respectively. The value of k_a(app) upon extrapolation to $T/\eta = 0$ is slightly positive, but is indistinguishable from zero within experimental error. An inverse dependence of k_a(app) on solution viscosity is necessary but not sufficient proof of a diffusion-controlled reaction (Schmitz & Schurr, 1972; Schurr & Schmitz, 1976). Still, it is consistent with the conclusion that the rate-limiting step that we measure under strong binding conditions is the bimolecular association of G32P to the polynucleotide to form the first non-co-operatively bound complexes. The rate constant for the formation of non-co-operatively bound G32P is specified as k_1 (hence under strong binding conditions, $k_a(\text{app}) = k_1$; see equations (4), (11) and Fig. 13).

(b) *Association behavior in the weak binding region*

(i) *Nucleic acid concentration dependence*

Upon performing the same set of association experiments as described above at constant [G32P] but with varying [poly(rU)] ([G32P] $\ll$ [poly(rU)]) in buffer T (0·1 M-NaCl, 25·0°C), we observed a quite different dependence of τ_f^{-1} on [poly(rU)] as shown in Figure 5. We have included the data for poly(dT) and poly(rϵA) in Figure 5 for comparison. These experiments were performed with identical concentrations of G32P (0·20 μM). In the poly(rU) case, τ_f^{-1} is initially directly proportional to [poly(rU)], but curvature is observed at higher [poly(rU)], where τ_f^{-1} seems to approach a plateau value. Over this same [poly(rU)] range, τ_s is essentially independent of [poly(rU)], as was the case for the other nucleic acid lattices. The dependence of τ_f^{-1} on [poly(rU)] is not consistent with a simple bimolecular association and indicates the presence of an intermediate along the kinetic pathway. Qualitatively similar behavior of τ_f^{-1} as a function of [NA] is also observed for the G32P–poly(rA) association under these same conditions. However, at the same nucleotide concentration, τ_f^{-1} poly(rA) $< \tau_f^{-1}$ poly(rU), which reflects some effects which are specific to the nucleic acid used.

Poly(rU) and poly(rA) have the weakest affinities for G32P of the lattices we have investigated (only poly(rC) is weaker; Newport *et al.*, 1981) although $K\omega$ should still be $> 10^7$ at 0·1 M-NaCl. We therefore measured the association rates for the other homopolynucleotides at [NaCl] $> 0{\cdot}10$ M (in order to decrease $K\omega$) to see

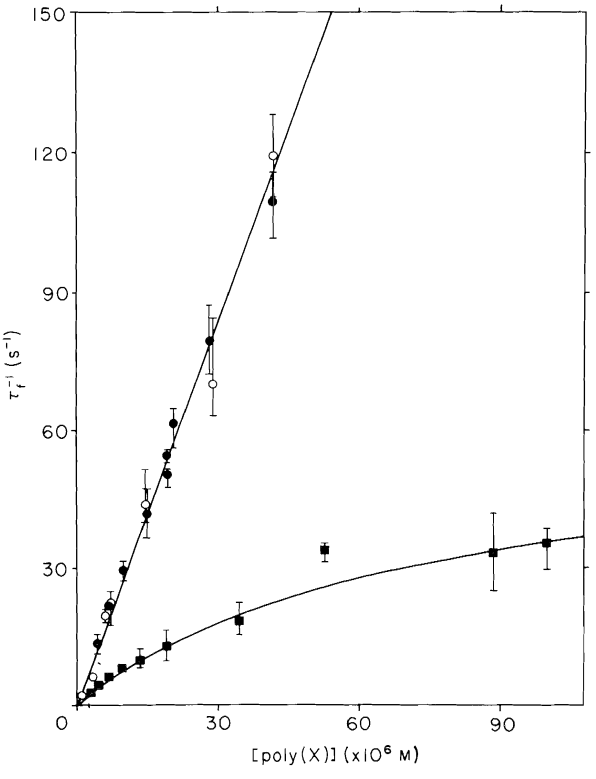

FIG. 5. τ_f^{-1} *versus* [poly(rU)] (■) in buffer T (pH 8·3), 0·10 M-NaCl, 25·0°C. The poly(dT) (●) and poly(rεA) (○) data from Fig. 2 are shown for comparison. The smooth curve through the poly(rU) data was generated using eqn (6) and the appropriate values of K_1 and τ_f (∞) ([G32P] = 0·19 μM).

if the qualitative behavior observed for poly(rU) and poly(rA) at 0·10 M is related to a lower binding affinity for G32P. In Figure 6 we show a series of experiments at [NaCl] ≥ 0·10 M for the G32P–poly(rεA) association. At [NaCl] > 0·10 M, the association rate decreases as the [NaCl] is increased. Recall that this [NaCl] dependence is the opposite of that observed at [NaCl] < 0·10 M where k_a(app) increases with increasing [NaCl] (see Fig. 4). At 0·20 M, 0·25 M and 0·30 M-NaCl we observe the same qualitative behavior as for poly(rU) and poly(rA) at 0·10 M-NaCl. τ_f^{-1} is initially directly proportional to [poly(rεA)] but appears to approach a plateau at high [poly(rεA)]. The onset of this non-linearity of τ_f^{-1} as a function of [NA] seems to appear under conditions which reduce the binding constant of G32P for the nucleic acid (e.g. at higher salt). Hence we refer to this type of behavior as weak binding association.

At 0·40 M-NaCl, τ_f^{-1} again appears to be directly proportional to [poly(rεA)] over the concentration range studied in Figure 6. Two questions arise at this point. Is the linear dependence of τ_f^{-1} on [NA], which is observed in the strong binding region, simply the result of making measurements over a limited concentration range? (i.e. does curvature become apparent only at concentrations $> 5 \times 10^{-5}$ M

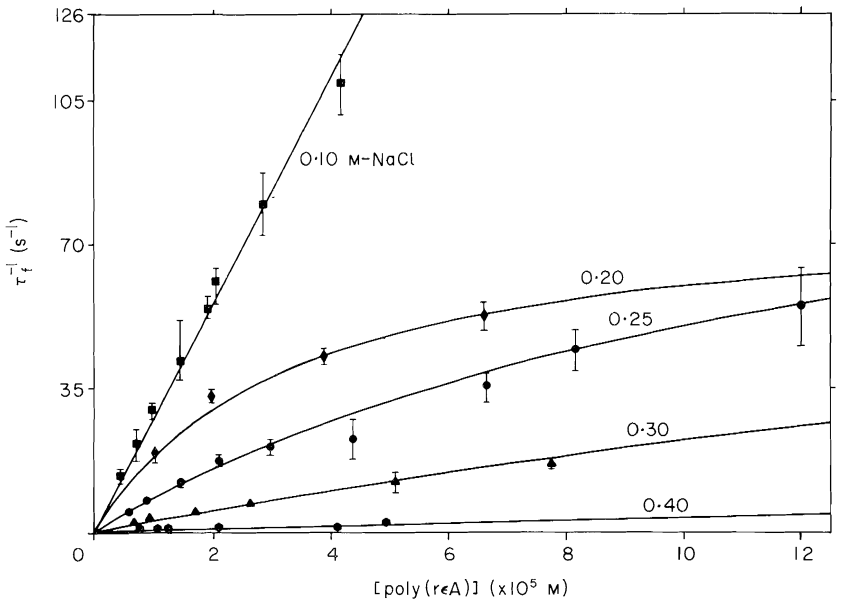

FIG. 6. [NaCl] dependence of the T4 G32P–poly(rεA) association under weak binding conditions ([NaCl] ≥ 0·20 M); 0·10 M-NaCl is a strong binding condition (buffer T, pH 8·3, 25·0°C). The smooth curves for the [NaCl] ≥ 0·20 M were generated using eqn (6) ([G32P] = 0·21 μM).

(nucleotides) for poly(dT), poly(rI) and poly(rεA) at 0·10 M-NaCl?; see Figure 2). Also, does the reappearance of a linear dependence of τ_f^{-1} on [poly(rεA)] at 0·40 M-NaCl indicate that we have re-entered the association conditions that are applicable to the strong binding region?

(ii) *G32P concentration dependence of the association rate*

We have examined the dependence of τ_f^{-1} on [G32P] under weak binding conditions (poly(rA), buffer T, 0·10 M-NaCl) with the [poly(rA)] in excess over the [G32P], as in the previous experiments. Figure 7(a) shows a plot of τ_f^{-1} *versus* total [G32P] at constant [poly(rA)]. Contrary to the results obtained under strong binding conditions, where τ_f^{-1} is independent of [G32P], we observe that τ_f^{-1} is directly proportional to the total [G32P] in the weak binding region, at the concentrations used, even though we are under what should be pseudo-first-order conditions. At the highest [G32P] in Figure 7(a), the final fractional saturation of the poly(rA) lattice is only 0·065. This same dependence of τ_f^{-1} on [G32P] is observed with all of the other nucleic acids, under the appropriate weak binding conditions. In Figure 7(b) we show the nucleic acid concentration dependence at three [G32P] for the association with poly(rεA) in buffer T (0·3 M-NaCl). Once again we observe the non-linear dependence of τ_f^{-1} on [poly(rεA)] at a constant [G32P] and τ_f^{-1} increases as the [G32P] is increased at a constant [poly(rεA)].

We now see that the weak binding region is also distinguished from the strong binding region by the dependence of τ_f^{-1} on [G32P] in addition to the non-linear

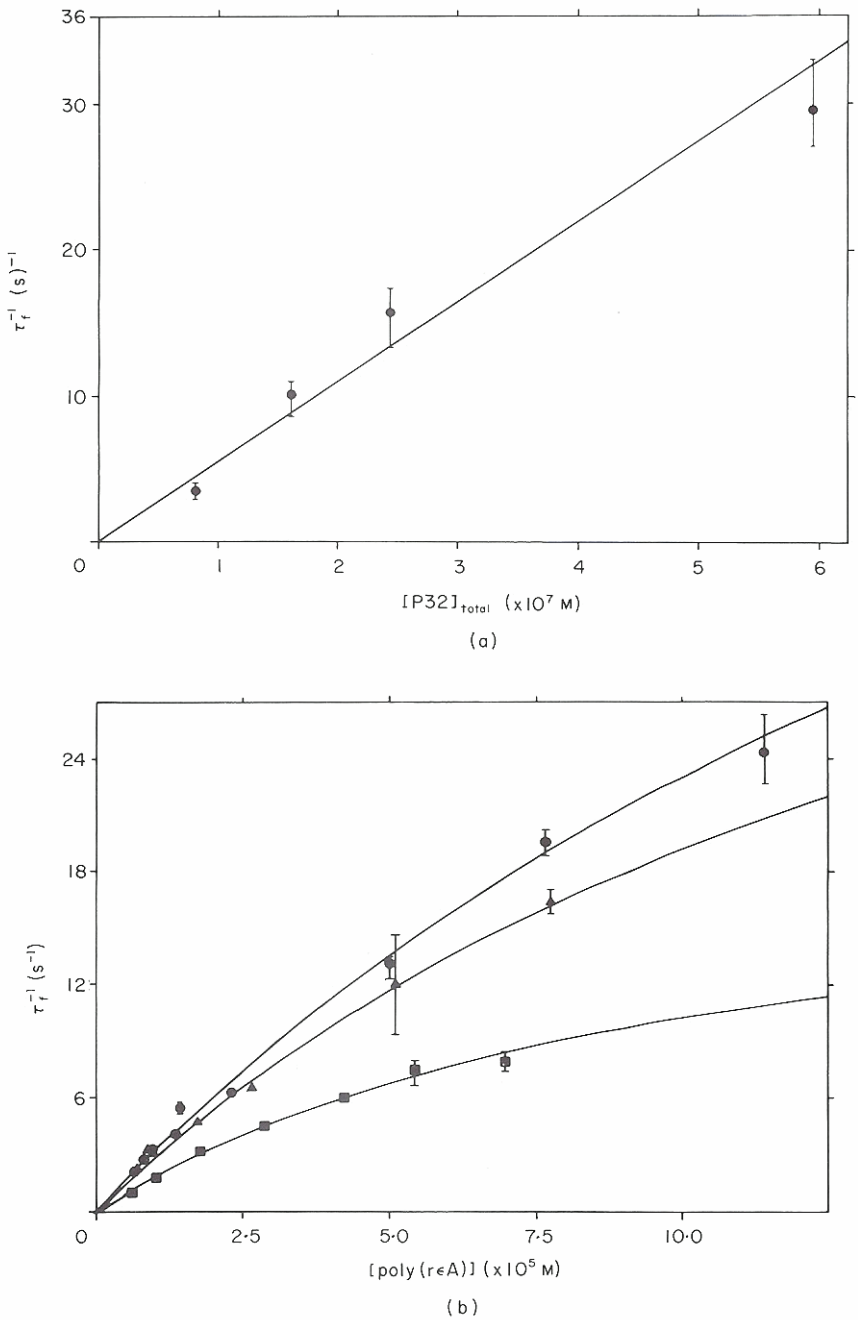

FIG. 7. (a) [G32P] dependence of τ_f^{-1} at a constant [poly(rA)] = $6{\cdot}4 \times 10^{-5}$ M (nucleotides) (buffer T, pH 8·3; 0·10 M-NaCl; 25·0°C). (b) Poly(rϵA) concentration dependence of τ_f^{-1} at 3 G32P concentrations (buffer T, 0·3 M-NaCl, 25·0°C). [G32P] = 0·10 μM (■); 0·21 μM (▲); 0·30 μM (●). The smooth curves were generated using eqn (6).

behavior of τ_f^{-1} as a function of [NA]. The [G32P] dependence of τ_f^{-1} is in fact the best diagnostic of weak binding association behavior. Hence, even though τ_f^{-1} is directly proportional to [poly(rεA)] at 0·4 M-NaCl and no curvature is detectable (see Fig. 6), the fact that τ_f^{-1} is dependent upon [G32P] indicates that 0·4 M-NaCl is a weak binding condition for the G32P–poly(rεA) association and curvature would be detected at higher [poly(rεA)].

(iii) *The association rate in the weak binding region exhibits nucleic acid specificity as well as the same salt dependence as the equilibrium constant*

The association rate measurements made with poly(dT) and poly(rI) show the same behavior at high [NaCl] as we observed with poly(rεA), i.e. curvature and apparent plateauing of τ_f^{-1} at high [NA], as well as a dependence on [G32P]. However, as with poly(rU) and poly(rA) at 0·1 M-NaCl, the magnitude of τ_f^{-1} at the same nucleotide and NaCl concentrations is quite dependent on the particular nucleic acid. In Figure 8, we show a series of association experiments at 0·3 M-NaCl in buffer T. We measured the dependence of τ_f^{-1} on [NA] for poly(dT), poly(dU), poly(rI) and poly(rεA), at a constant [G32P] and found a quantitative difference among the relaxation times for the four lattices. The association rate is greatest for G32P binding to poly(dT) and decreases in the order, poly(dT) > poly(dU) > poly(rI) > poly(rεA) > poly(rU) > poly(rA). (The poly(rU) and poly(rA) data were obtained at 0·10 M-NaCl.) This specificity is the same as that observed for $K\omega$ (Newport *et al.*, 1981). Recall that no such specificity was observed among poly(dT), poly(rI) and poly(rεA) under strong binding conditions.

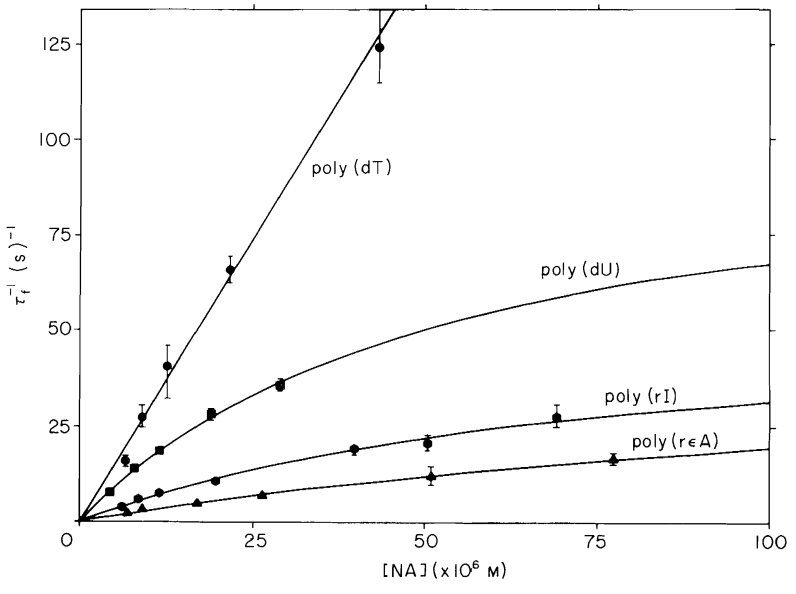

FIG. 8. Nucleic acid specificity under weak binding conditions. Nucleic acid concentration dependence of τ_f^{-1} at constant [G32P] = 0·21 μM. The smooth curves for all but poly(dT) were generated using eqn (6).

In Figure 4 we have plotted k_a(app) *versus* [NaCl] on a log–log scale for poly(dT), poly(rI) and poly(rεA). Under these weak binding conditions where a non-linear dependence of τ_f^{-1} on [NA] is observed, the values of k_a(app) were calculated from reciprocal plots of τ_f *versus* $[NA]^{-1}$ as shown in Figure 9. The inverse of the slope of such a reciprocal plot is k_a(app). This is a convenient way to represent the weak binding data; however, as shown below, it does not represent a true rate constant (see sub-section (iv), below, for the definition of k_a(app) in the weak binding region). The specificity under weak binding conditions is readily apparent in Figure 4, as well as the lack of specificity under strong binding conditions (generally $\leq 0{\cdot}10$ M-NaCl). There is a broad optimum for k_a(app) in the range $0{\cdot}1\ \text{M} < [\text{NaCl}] < 0{\cdot}20$ M, although the breadth of this optimum depends on the homopolynucleotide. In addition, we see that the [NaCl] at which the association reaction moves from the strong binding to the weak binding range is also dependent upon the homopolynucleotide. For the association with poly(rεA), the weak binding range begins $\sim 0{\cdot}15$ M-NaCl, whereas at 0·3 M-NaCl, the poly(dT) association still exhibits strong binding behavior. Hence the strong binding and weak binding regions are different for each nucleic acid. The values of $(\partial \log k_a(\text{app})/\partial \log [\text{NaCl}])$ for poly(dT), poly(rI) and poly(rεA), in the linear range at high salt (see Fig. 4) are $-3{\cdot}6 \pm 0{\cdot}5$, $-5{\cdot}0 \pm 0{\cdot}8$, and $-4{\cdot}9 \pm 0{\cdot}8$, respectively. Although we have shown (Fig. 7) that the absolute magnitude of k_a(app) is dependent upon [G32P], under weak binding conditions, the [NaCl] dependence of k_a(app) is independent of [G32P] as long as the same [G32P] is used at each [NaCl]. These values of $\partial \log k_a(\text{app})/\partial \log [\text{NaCl}]$ compare well with the values of $\partial \log K\omega/\partial \log [\text{NaCl}] = -6 \pm 1$ obtained for poly(rεA) and poly(rI) as well as a number of other homopolynucleotides and $-3{\cdot}2 \pm 0{\cdot}5$ for poly(dT) (Kowalczykowski *et al.*, 1981*b*; Newport *et al.*, 1981).

(iv) *The weak binding association involves a pre-equilibrium formation of non-co-operatively bound G32P–nucleic acid complexes*

Based on the G32P concentration dependence, the salt dependence and the identical nucleic acid specificities followed by k_a(app) and $K\omega$ in the weak binding region, it seems that k_a(app) is related to the equilibrium constant for the interaction. In addition, the non-linear dependence of τ_f^{-1} on [NA] resembles an equilibrium titration curve (see Fig. 6) for the non-co-operative binding of a protein to a nucleic acid lattice. We have analyzed the [NA] dependence of τ_f^{-1} as a titration curve to obtain estimates of the equilibrium constants for isolated (non-co-operative) binding of G32P to the various homopolynucleotides. Our model is as follows. We have assumed that a rapid pre-equilibrium is established among free G32P (P), free DNA sites (D), and non-co-operatively bound G32P (PD_{is}; for isolated protein–DNA complexes). This can be written as:

$$P + D \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} PD_{is}, \tag{4a}$$

$$PD_{is} + P \xrightarrow{k_2} PD_{cluster}, \tag{4b}$$

where step (4a) represents the pre-equilibrium formation of PD_{is} with equilibrium constant $K_1 = k_1/k_{-1}$ and step (4b) represents all subsequent (unspecified) steps for the formation of co-operatively bound G32P clusters. As we will show below, the concentration of PD_{is} formed in the pre-equilibrium (step (4a)) is quite low under weak binding conditions. In addition, the rate of formation of PD_{is} should be fast so that this step will be difficult to detect in the stopped-flow experiment (under weak binding conditions). Once the PD_{is} are established, however, they will act as nucleation sites for the formation of contiguously bound protein molecules and the co-operative interactions that stabilize them (step (4b)). We assume that τ_f detects the formation of the first contiguously bound protein molecules. From (4b) it follows that:

$$-\frac{d[P]}{dt} = 2k_2[P][PD_{is}]$$

and (5)

$$-\frac{1}{[P]}\frac{d[P]}{dt} = \tau_f^{-1} = 2k_2[PD_{is}].$$

The result from equation (5) is that τ_f^{-1} is proportional to $[PD_{is}]$. (The factor of 2 in equation (5) is needed because we define k_2 as the association rate constant to a singly contiguous site in units of M^{-1} (contiguous site) s^{-1} and each non-co-operatively bound protein (PD_{is}) possesses two singly contiguous sites.) If this is the case, then τ_f^{-1} can be used as a signal that is sensitive to the formation of PD_{is} (similar to an optical change upon formation of a complex). Since the $[NA] \gg [G32P]$ in all cases, we can neglect contributions due to overlap of potential nucleic acid binding sites and treat the binding as if it were to independent nucleic acid sites. The non-co-operative binding constant is defined as:

$$K_1 = \frac{[PD_{is}]}{[P]D_T},$$

where D_T is the total concentration of nucleotides. We further define $\tau_f^{-1}(\infty)$ as the value of τ_f^{-1} in the limit of very high nucleic acid concentration (i.e. the plateau value of τ_f^{-1} that would be obtained if the total G32P in a reaction, (P_{tot}), would initially bind in the non-co-operative mode; $\tau_f^{-1}(\infty) = 2k_2P_{tot}$). With these definitions we obtain:

$$\frac{\tau_f^{-1}}{\tau_f^{-1}(\infty)} = \frac{[PD_{is}]}{P_{tot}} = \frac{K_1D_T}{1+K_1D_T}, \tag{6}$$

which rearranges to:

$$\tau_f = \frac{\tau_f(\infty)}{K_1}\frac{1}{D_T} + \tau_f(\infty). \tag{7}$$

Therefore, a reciprocal plot of τ_f *versus* D_T^{-1} should be linear. The value of K_1 is obtained from a ratio of the intercept/slope. It is obvious from equation (7) that our definition of k_a(app), in the weak binding region given in the previous section as the inverse of the slope of a reciprocal plot, yields $k_a(\text{app}) = \tau_f^{-1}(\infty)K_1$, and hence does not constitute a true rate constant.

A reciprocal plot of the G32P–poly(rϵA) association data at 0·25 M-NaCl is shown in Figure 9, where we see that τ_f is a linear function of $([\text{poly(r}\epsilon\text{A)}])^{-1}$. The equilibrium constant for the isolated, non-co-operative binding of G32P to poly(rϵA) as determined from Figure 9 is $K_1 = 7{\cdot}7 \pm 1 \times 10^3\ \text{M}^{-1}$ (0·25 M-NaCl, pH 8·3, 25·0°C). Using equation (6) and the values of K_1 and τ_f (∞) obtained from the reciprocal plots, we have generated the theoretical curves for the τ_f^{-1} *versus* [NA] plots which are shown in Figures 5, 6, 7 and 8. In all cases the theoretical fit to the data is quite good and reproduces all the observed qualitative features.

In Table 2 we have listed the non-co-operative equilibrium constants, K_1, for various homopolynucleotide–G32P interactions as determined from the kinetic data at a number of solution conditions. The most serious source of error in these estimates is the determination of the correct endpoint (i.e. $\tau_f^{-1}(\infty)$, obtained from the intercept of the reciprocal plot; see Fig. 9). This is most difficult for the experiments at the highest salt concentrations, where binding is weakest. The endpoint, as in all titrations, is most accurately determined when data can be obtained in the region where curvature is observed (i.e. saturation is being approached). At the lower [NaCl], (e.g. 0·20 M for poly(rϵA); Fig. 6) the endpoint is most easily determined, since K_1 is larger and distinct curvature in the τ_f^{-1} *versus* [poly(rϵA)] plot is observed. One would have to go to much higher (inaccessible) [poly(rϵA)] to see curvature at 0·40 M-NaCl. This is why the τ_f^{-1} *versus* poly(rϵA) plots appear linear at very high [NaCl]. We have therefore used the endpoint determined at lower [NaCl] (0·20 M and 0·25 M) to fit the 0·40 M-NaCl data. This should be more accurate than attempting to estimate an endpoint from the 0·40 M-NaCl data alone.

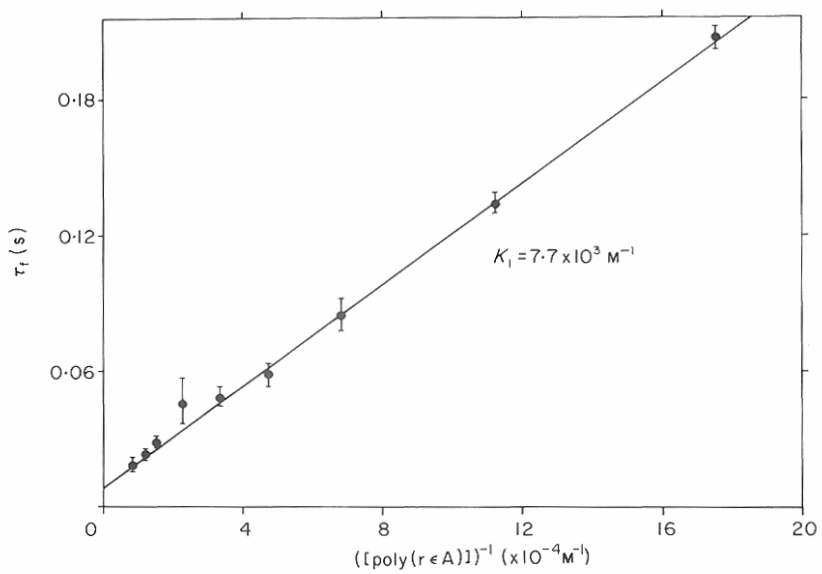

FIG. 9. Reciprocal plot (τ_f *versus* $[\text{NA}]^{-1}$) for the T4 G32P–poly(rϵA) association (buffer T, 0·25 M-NaCl, 25·0°C) at constant [G32P] = 0·21 μM.

TABLE 2

Non-co-operative equilibrium constants for G32P

Polynucleotide	[Salt]	[G32P] ($\times 10^7$ M)	K_1(M^{-1})†
Poly(rU)	0·10 M-NaCl	2·0	$1{\cdot}5 \pm 0{\cdot}5 \times 10^4$
	0·10 M-NaF	2·1	$1{\cdot}5 \pm 0{\cdot}5 \times 10^4$
Poly(rA)	0·10 M-NaCl	2·1	$1{\cdot}5 \pm 0{\cdot}5 \times 10^4$
	0·20 M-NaCl	2·1	$4{\cdot}1 \pm 1{\cdot}0 \times 10^3$
Poly(rεA)	0·20 M-NaCl	2·1	$3{\cdot}3 \pm 1{\cdot}0 \times 10^4$
	0·25 M-NaCl	2·1	$7{\cdot}7 \pm 2{\cdot}0 \times 10^3$
	0·30 M-NaCl	2·1	$2{\cdot}4 \pm 1{\cdot}0 \times 10^3$
	0·40 M-NaCl	2·1	$7{\cdot}5 \pm 2{\cdot}0 \times 10^2$
	0·30 M-NaF	2·1	$1{\cdot}5 \pm 0{\cdot}5 \times 10^4$
Poly(dU)	0·30 M-NaCl	2·1	$2{\cdot}0 \pm 0{\cdot}7 \times 10^4$
Poly(rI)	0·30 M-NaCl	2·1	$1{\cdot}0 \pm 0{\cdot}5 \times 10^4$
Poly(dT)	0·46 M-NaCl	1·3	$2{\cdot}1 \pm 1{\cdot}0 \times 10^4$
	0·60 M-NaCl	4·1	$7{\cdot}3 \pm 2{\cdot}0 \times 10^3$
	0·90 M-NaCl	4·1	$1{\cdot}9 \pm 0{\cdot}5 \times 10^3$

† K_1 was determined from a reciprocal plot of τ_f *versus* $[NA]^{-1}$ as analyzed using equation (7) in the text.

The values of K_1 determined from the kinetic data are independent of the total G32P concentration used (to within $\pm 50\%$). They also agree very well with the values of K determined by Kowalczykowski *et al.* (1981*b*) and Newport *et al.* (1981) from equilibrium titrations of homopolynucleotides with G32P. They measured $K\omega$ for the co-operative binding of G32P, and were also able to obtain separate estimates of K and ω. The estimates range between $10^3 < \omega < 10^4$ with the average values being 2×10^3 to 5×10^3. The value of ω was also found to be fairly independent of salt concentration, with the salt dependence of $K\omega$ residing in K (Kowalczykowski *et al.*, 1981*b*; Newport *et al.*, 1981). In the equilibrium measurements, the product $K\omega$ is more accurately determined than either parameter separately, whereas the kinetic experiments yield a direct determination of K_1. To facilitate a comparison between the values of K_1 obtained here and those obtained by Kowalczykowski *et al.* (1981*b*) and Newport *et al.* (1981), we have determined the value of ω that is needed to make them equivalent.

At this point we note that although the units of K_1 (M^{-1} (nucleotides)) and K (obtained from $K\omega$; M^{-1} (protein)) may appear different, the fact that each nucleotide is the start of a potential binding site for each protein indicates that the values can be directly compared. However, the two estimates of K (from $K\omega$) and K_1 are such that they do not generally overlap. The kinetic estimates are at lower salt concentrations and hence higher values of K_1. Therefore, the comparisons have been made by extrapolation of the log K − log [NaCl] plots from the higher [NaCl] region of Kowalczykowski *et al.* (1981*b*) and Newport *et al.* (1981) to the lower [NaCl] region presented here. For a protein–nucleic acid interaction, a log K − log [NaCl] plot is predicted to be linear if the solution contains only monovalent salt as the supporting electrolyte and in the absence of both ion release from the protein and severe preferential hydration of the macromolecules. The

reader is referred to Record *et al.* (1976,1978) for a discussion of such plots and their interpretation.

In Figure 10 we compare the two estimates of K_1 for the interaction of G32P with poly(rA) and poly(rεA). For both nucleic acids, a value of $\omega = 3{\cdot}5 \times 10^4$ was used to obtain K from the $K\omega$ estimates of Newport *et al.* (1981) and Kowalczykowski *et al.* (1981*b*). The values of ω needed to obtain good agreement between the kinetic and equilibrium determinations of K_1 for the other polynucleotides are all in the range $1 \times 10^4 \leq \omega \leq 3{\cdot}5 \times 10^4$ with the exception of poly(dT)† which requires $\omega \sim 4 \times 10^3$. These values of ω are all slightly higher (a factor of 7 to 10) than the average estimates of Newport *et al.* (1981), although some of their estimates were as high as 1×10^4. As noted by Kowalczykowski *et al.* (1981*b*), their procedure for the estimation of ω may represent a lower limit. Since they use the infinite lattice approximation to interpret their data, any significant population of polynucleotides that are not long enough to approximate an infinite lattice will result in a low estimate for ω, although the product $K\omega$ should still be quite

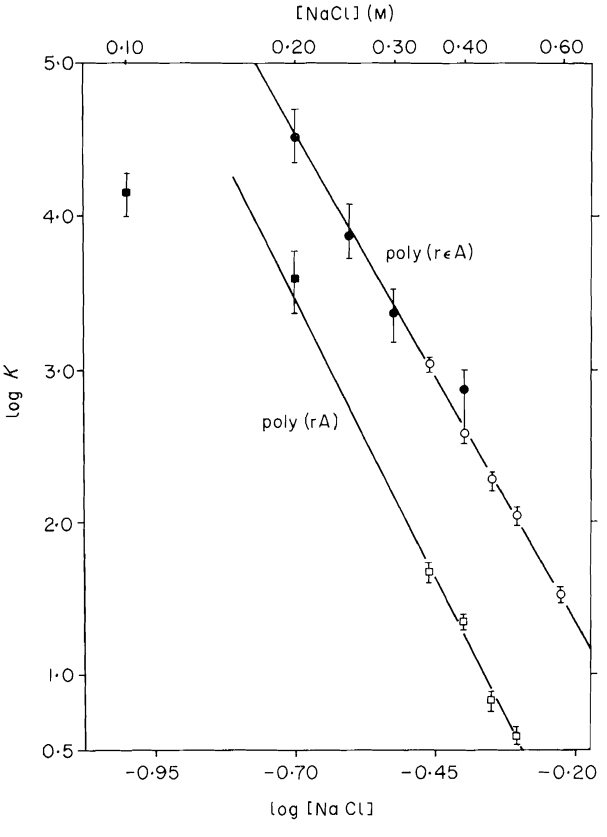

FIG. 10. [NaCl] dependence of the non-co-operative equilibrium constant K_1 (log–log plot). Filled symbols, kinetic determination of K_1 using eqn (7). Open symbols, values from Kowalczykowski *et al.* (1981*b*) and Newport *et al.* (1981), assuming a value of $\omega = 3{\cdot}5 \times 10^4$.

accurate. As a result, the kinetic estimates of K_1 presented here when compared to the product $K\omega$, as determined by Kowalczykowski *et al.* (1981*b*) and Newport *et al.* (1981), may provide a more accurate estimate of ω. This comparison suggests that ω may be much higher than 10^3 and possibly as high as 1×10^4 to 3×10^4. In addition to the agreement found between the kinetic and equilibrium estimates of K_1 shown in Figure 10, we also observe excellent agreement between the [NaCl] dependences of the two sets of data for poly(rεA). A value of $\partial \log K_1/\partial \log [\text{NaCl}] = -6{\cdot}4$ provides a good fit to all of the poly(rεA) data over the range 0·20 M $\leq$ [NaCl] $\leq$ 0·60 M. Recalling that $\partial \log K\omega/\partial \log [\text{NaCl}] = -6 \pm 1$ for poly(rεA) (Newport *et al.*, 1981) this lends further support to the conclusion of Kowalczykowski *et al.* (1981*b*) and Newport *et al.* (1981) that ω is independent of [NaCl]. This also indicates that in the proposed intermediate, PD_{is}, G32P binds to the nucleic acid in the polynucleotide mode rather than in the oligonucleotide mode since the salt dependence of G32P binding in the oligonucleotide mode is only $\tilde{-}1{\cdot}0$ (Kowalczykowski *et al.*, 1981*b*).

The value of K_1 for poly(rA) at 0·20 M-NaCl (Fig. 10) does follow the extrapolation from the higher [NaCl] data, although the value at 0·10 M-NaCl falls below the extrapolated estimate. This deviation from linearity in the log K − log [NaCl] plot may result from either a change in ω, or from a change in the number of ions released by G32P when it interacts with the nucleic acid at low salt. The former seems unlikely since ω has been shown to be independent of salt concentration at high [NaCl] (Kowalczykowski *et al.*, 1981*b*; Newport *et al.*, 1981). Furthermore, estimates of ω (at 0·01 M-NaCl) obtained from studies of the perturbation of the helix–coil transition of poly(dA·T) by G32P are in good agreement with the high salt estimates (Jensen *et al.*, 1976). Newport *et al.* (1981) have shown that there is a substantial contribution to the salt dependence of $K\omega$ due to release of anions from the G32P. If the anion binding to the G32P is governed by mass action (as opposed to a condensation effect as in cation binding to DNA; see Manning, 1978; Record *et al.*, 1976,1978), then there should be fewer anions bound and hence fewer anions released when G32P binds to nucleic acids at low salt. This is qualitatively what is observed for the G32P–poly(rA) and poly(rU) interactions. Kinetic estimates of K_1 at 0·1 M-NaCl are only available for poly(rA) and poly(rU) (all other lattices, except poly(rC), are in the strong binding limit at 0·10 M-NaCl); however, it seems highly probable that the binding constants for the other nucleic acids will also deviate from their high salt extrapolation at 0·10 M-NaCl.

The calculations presented above support the idea that the first step in the association mechanism under weak binding conditions is the pre-equilibrium formation of non-co-operatively bound (isolated) G32P–nucleic acid complexes.

†The equilibrium values of $K\omega$ for poly(dT) were obtained at quite high salt concentrations (>2 M-NaCl; Newport *et al.*, 1981) and hence require a large extrapolation into the [NaCl] range used in the kinetics experiments. This long extrapolation increases the inaccuracy in the estimates of K in the low [NaCl] range and is most likely the reason for the lower value of ω. As a result, the kinetic estimates of K_1 for poly(dT) should be more accurate in the low [NaCl] range. Since individual estimates of ω for poly(dT) were not obtained by Newport *et al.* (1981), we cannot eliminate the possibility that ω is lower for poly(dT).

These non-co-operatively bound G32P molecules presumably act as nucleation sites for the formation of co-operatively bound protein clusters.

(v) *Amplitudes*

Although we have not undertaken a complete study of the amplitudes of the relaxation times and their dependence upon nucleic acid concentration, we have made some measurements that pertain to the mechanism of the G32P–polynucleotide interaction. A series of amplitude measurements were made under weak binding conditions at constant [G32P] = 0·21 μM and varying [poly(rA)], in buffer T (0·10 M-NaCl, 25·0°C). At low [poly(rA)] (e.g. $< 2 \times 10^{-5}$ M (nucleotides)), the decrease in G32P fluorescence (ΔF) observed in the association experiment is $100 \pm 5\%$ of the ΔF observed in an equilibrium titration experiment, hence we detect essentially all of the fast phase that has a ΔF associated with it. As the [poly(rA)] is increased, the amplitude of τ_f^{-1} decreases. Within experimental error, the percentage of the amplitude that is missed (occurring within the instrument dead time) is equal to the percentage of G32P that we calculate would bind to poly(rA), under these conditions, if G32P could bind only in the non-co-operative mode. This is in agreement with the model and results discussed above, supporting the existence of a fast pre-equilibrium formation of non-co-operatively bound G32P as the first step in the weak binding mechanism. The reason that essentially all of the fluorescence change is observed at low nucleic acid concentrations is that $< 5\%$ of the G32P would bind in the non-co-operative mode under these conditions, and hence very little signal is missed.

(vi) τ_f^{-1} *is proportional to the concentration of non-co-operatively bound G32P* (PD_{is}) *and is independent of free* [*G32P*]

Using the values of K_1 we can calculate the concentration of non-co-operatively bound G32P ($[PD_{is}]$) formed in the pre-equilibrium complex. With this we can check our assumption that τ_f^{-1} is proportional to $[PD_{is}]$ (see eqn (5)). In Figure 11(a) we have plotted τ_f^{-1} *versus* $[PD_{is}]$ for the G32P–poly(rϵA) association at 0·25 M-NaCl. The concentrations of the non-co-operatively bound G32P were calculated using the isotherm of McGhee & von Hippel (1974) (eqn (10) of their paper) for the binding of a ligand that covers $n = 7$ nucleotides. A binding constant of $K_1 = 7{\cdot}7 \times 10^3$ M^{-1}, obtained from the reciprocal plot (Fig. 9) was used in the calculation. From Figure 11(a) we see that τ_f^{-1} is directly proportional to $[PD_{is}]$ over the entire concentration range studied. This is consistent with our hypothesis that the non-co-operatively bound G32P molecules act as nucleation sites for the subsequent formation of protein clusters. This direct proportionality between τ_f^{-1} and $[PD_{is}]$ exists for all of the weak binding association experiments we have performed under all conditions and with all polynucleotides.

The direct proportionality between τ_f^{-1} and $[PD_{is}]$ is also independent of the free [G32P]. In Figure 11(b) we show association experiments at two different total protein concentrations. The values of τ_f^{-1} from both sets of experiments fall on a common line when plotted *versus* $[PD_{is}]$. This indicates that the relaxation time,

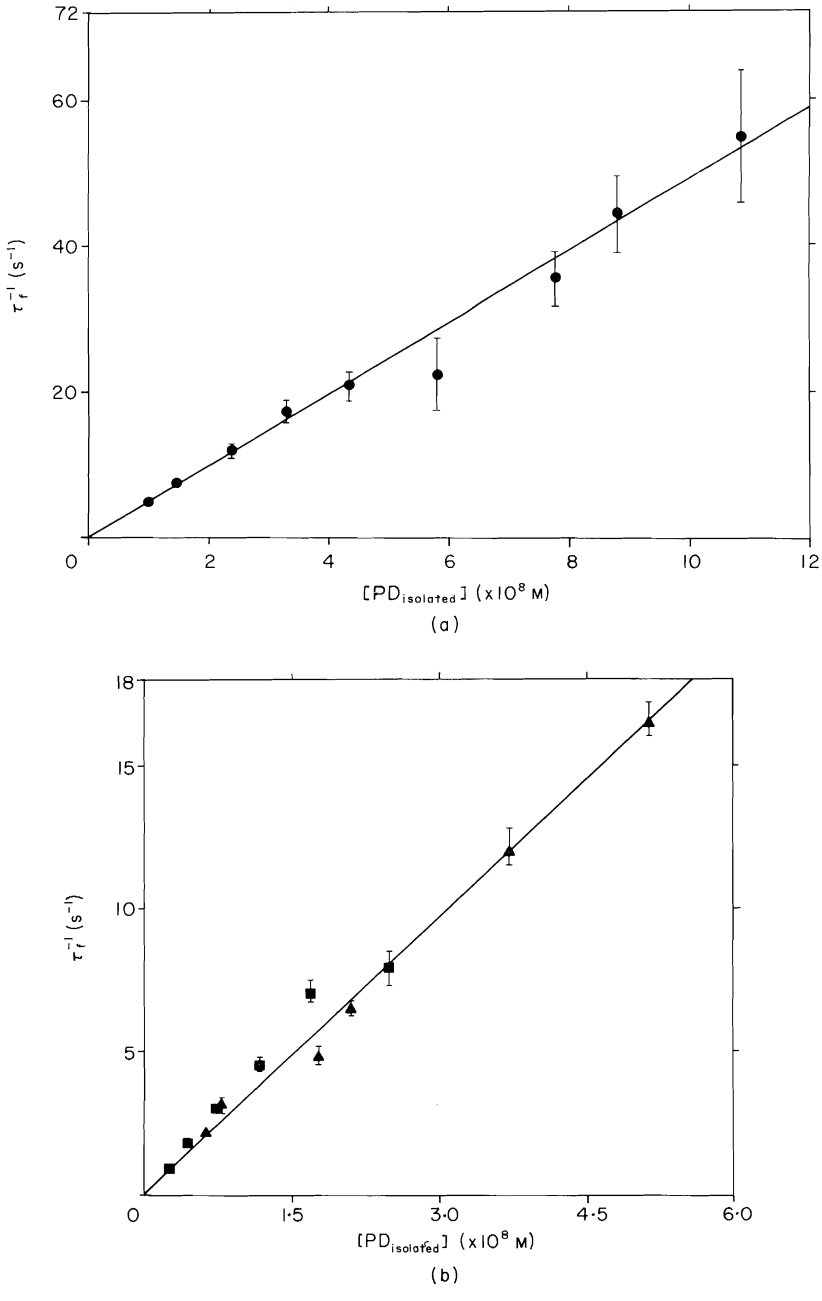

FIG. 11. τ_f^{-1} is directly proportional to the non-co-operatively bound [G32P]($PD_{isolated}$) which is formed in the fast pre-equilibrium step and independent of the free [G32P]. (a) G32P–poly(rεA) association at 0·25 M-NaCl, 25·0°C, buffer T. The [$PD_{isolated}$] was calculated using eqn (10) of McGhee & von Hippel (1974), using a site size $n = 7$ and $K_1 = 7{\cdot}7 \times 10^3$ M^{-1}. $k_2 = \frac{1}{2}(\partial\tau_f^{-1}/\partial[PD_{is}]) = 2{\cdot}5 \times 10^8$ M^{-1} (contiguous site) s^{-1}. (b) G32P–poly(rεA) association in buffer T, 0·30 M-NaCl, 25·0°C. [G32P] = 0·21 μM (▲) and 0·10 μM (■); $k_2 = 2{\cdot}0 \times 10^8$ M^{-1} (contiguous site) s^{-1}.

τ_f^{-1}, is dependent only upon the concentration of PD_{is} and is independent of the free G32P concentration. (The *rate* of association of G32P is dependent upon the free protein concentration; see equations (5).) This also constitutes the best evidence in support of our assumption that $\tau_f^{-1} \propto [PD_{is}]$. For example, in Figure 11(b) at $[PD_{is}] = 7{\cdot}8 \times 10^{-9}$ M, $\tau_f^{-1} = 3\ s^{-1}$ for both sets of data, even though the free [G32P] differs by more than a factor of two. (Note that the same $[PD_{is}]$ is obtained only by having different total [poly(rεA)] for these two experiments.) Hence, τ_f^{-1}, in the weak binding association mechanism, is dependent only upon the concentration of non-co-operatively bound G32P, which is established in the pre-equilibrium step. The effect of increasing [G32P], even in the presence of excess [NA], is now apparent. Upon increasing the [G32P], more non-co-operatively bound G32P is formed in the pre-equilibrium step (nucleation). Since τ_f^{-1} is proportional to $[PD_{is}]$ (growth step), an increase in the rate of association is observed due to an increase in nucleation sites.

In Table 3 we have listed the slopes obtained from plots of τ_f^{-1} *versus* $[PD_{is}]$ for the various polynucleotides at several different solution conditions. (Table 3 actually lists values of

$$k_2 = 1/2 \frac{\partial \tau_f^{-1}}{\partial [PD_{is}]};$$

see eqn (5).) The slopes are sensitive to [NaCl] and temperature. For poly(rεA) at 25·0°C and 0·25 M-NaCl (the conditions of Fig. 11(a)), the slope is

TABLE 3

Rate constant for the growth step (k_2)

Polynucleotide	[Salt]	Temperature (deg. C)	k_2($\times 10^{-8}$ M^{-1} s^{-1})†
Poly(rεA)	0·20 M-NaCl	25·0	1·8
	0·25 M-NaCl	25·0	2·5
	0·30 M-NaCl	25·0	2·0
	0·40 M-NaCl	25·0	1·4
	0·30 M-NaF	25·0	2·6
Poly(rU)	0·10 M-NaCl	25·0	1·6
	0·10 M-NaF	25·0	1·5
Poly(rA)	0·10 M-NaCl	25·0	0·4
Poly(rI)	0·30 M-NaCl	25·0	1·8
Poly(dU)	0·30 M-NaCl	25·0	2·5
Poly(dT)	0·46 M-NaCl	25·0	2·1
	0·60 M-NaCl	25·0	1·7
	0·90 M-NaCl	25·0	1·35
Poly(rεA)	0·10 M-NaCl	10·0	3·6
	0·10 M-NaCl	12·0	11·0
	0·10 M-NaCl	14·8	11·0
	0·10 M-NaCl	17·0	16·0
	0·10 M-NaCl	20·0	31·0

† k_2 has units of M^{-1} (contiguous site) s^{-1} and is obtained from a plot of τ_f^{-1} *versus* $[PD_{is}]$ using equation (5) in the text; $k_2 = \frac{1}{2}(\partial \tau_f^{-1}/\partial [PD_{is}])$.

5×10^8 M^{-1} (PD_{is}) s^{-1} ($k_2 = 2{\cdot}5 \times 10^8$ M^{-1} (contiguous site) s^{-1}). The interpretation of these rate constants will be discussed below.

(vii) *Effect of temperature on the weak binding association*

We have measured the effect of temperature on the G32P–poly(rεA) association in buffer H (0·10 M-NaCl). At 25·0°C, 0·10 M-NaCl, the G32P–poly(rεA) association displays strong binding behavior and $k_1 = 3{\cdot}0 \pm 0{\cdot}2 \times 10^6$ M^{-1} (nucleotide) s^{-1}, as shown above. However, as the temperature is lowered, the G32P–poly(rεA) association begins to display pre-equilibrium behavior as in the weak binding cases just discussed. In Figure 12, we have plotted τ_f^{-1} *versus* [poly(rεA)], from which one can see the transition from pre-equilibrium behavior in the region $T \leq 20$°C to strong binding behavior at $T \geq 25{\cdot}0$°C. In Table 4, the values of K_1, estimated from double reciprocal plots, are given for the $T \leq 20{\cdot}0$°C data. A van't Hoff plot yields $\Delta H^0_{obs} = -24 \pm 6$ kcal/mol for the binding of G32P to poly(rεA) in the non-co-operative mode. Kowalczykowski *et al.* (1981*b*) measured $\Delta H^0_{obs} = -22{\cdot}5 \pm 2{\cdot}5$ kcal/mol at 0·35, 0·40 and 0·45 M-NaCl for the co-operative interaction (i.e. $K\omega$) of G32P with poly(rεA). Their data seem to indicate a slight temperature dependence for ω; however, since it is difficult to separate K from ω

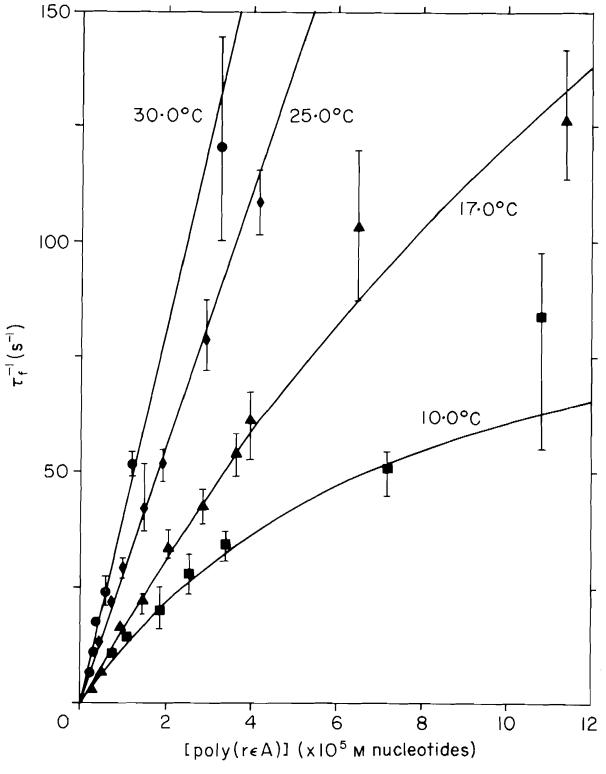

FIG. 12. Temperature dependence of the G32P–poly(rεA) association in buffer H, 0·10 M-NaCl. The [poly(rεA)] dependence of τ_f^{-1} ([G32P] = 0·21 μM).

TABLE 4

G32P–poly(rεA) temperature dependence (0·1 M-NaCl, 10 mM-HEPES, pH 7·5)

Temperature (deg. C)	$K_1(\times 10^{-3}\ \text{M}^{-1})$
20·0	2·8
17·0	4·1
14·8	7·4
12·0	4·7
10·0	13·0

$\Delta H^0_{obs} = 24 \pm 6$ kcal/mol.
$\Delta G^0_{obs} = -4{\cdot}6$ kcal/mol (20·0°C).
$\Delta S^0_{obs} = 66$ e.u. (20·0°C).

with great accuracy, this conclusion is not firm. Within experimental error, the ΔH^0_{obs} that we have determined for the non-co-operative G32P–poly(rεA) interaction is the same as the ΔH^0_{obs} for the co-operative interaction. This indicates that ω should have a very small temperature coefficient.

There is an apparent inconsistency between the values of K_1 determined at 0·10 M-NaCl, $T \leq 20{\cdot}0$°C and the 25·0°C data obtained at [NaCl] $\geq$ 0·20 M, in that the low temperature estimates of K_1 are much lower than expected. An extrapolation of the high salt values of K_1 yields $K_1 \sim 10^5\ \text{M}^{-1}$ at 0·10 M-NaCl, 25·0°C, whereas an extrapolation of the data from $T \leq 20{\cdot}0$°C yields $K_1 = 2 \times 10^3\ \text{M}^{-1}$ under the same conditions. We have no explanation for this apparent discrepancy.

(viii) *Effect of anions*

We have measured the effects of substituting NaF for NaCl on the association rate in the weak binding, pre-equilibrium region. Measurements were made on the association of G32P with poly(rU) at 0·1 M-NaF and 0·1 M-NaCl and with poly(rεA) at 0·3 M-NaF and 0·3 M-NaCl. The effects of fluoride were different for the two systems. Within experimental error there is no effect on the poly(rU) association; the [NA] dependences of τ_f^{-1} are identical as are the equilibrium constants, K_1 (see Table 2).

On the other hand, the G32P–poly(rεA) association in 0·3 M-NaF is a factor of four to five faster than in 0·3 M-NaCl. This difference is reflected in the calculated values of K_1; K_1(0·3 M-NaCl) $= 2{\cdot}4 \pm 1 \times 10^3\ \text{M}^{-1}$, K_1(0·3 M-NaF) $= 1{\cdot}5 \pm 0{\cdot}5 \times 10^4\ \text{M}^{-1}$. Newport *et al.* (1981) have observed a large dependence of $K\omega$ on anion type ($K\omega(F^-) > K\omega(Cl^-)$); however, they did not attempt to separate K from ω in their anion studies. Newport *et al.* (1981) did find a decrease in $|\partial \log K\omega / \partial \log [NaX]|$ in going from chloride to acetate to fluoride, indicating that the differential effects of anions decrease with decreasing anion concentration. We have also observed this trend in our studies of the dissociation of G32P–polynucleotide complexes (Lohman, unpublished results). Our kinetic estimates of K_1 indicate that there is an effect of the type of anion on K_1 at 0·3 M, but very little, if any, effect at 0·1 M. This is consistent with the studies mentioned above assuming a mass action description of anion binding to G32P.

4. Discussion

(a) *Association mechanism under strong binding conditions*

The data obtained under strong binding conditions are consistent with a simple bimolecular association mechanism. The rate-limiting step seems to be the non-specific association of G32P with the polynucleotide to form non-co-operatively bound (isolated) complexes (k_1 in equations (4), (11) and Fig. 13).

The value of $k_1 = 3 \times 10^6$ M^{-1} (nucleotide) s^{-1}, determined from Figure 2 at 0·10 M-NaCl, may appear to be too small to be a diffusion-controlled rate constant. Note, however, that k_1 is the non-co-operative association rate constant to a nucleotide (each nucleotide represents the start of a potential protein binding site). In the case of a diffusion-controlled reaction between a protein and a polynucleotide, the diffusing species are not individual nucleotides, but rather the entire polynucleotide chain. Therefore in order to obtain a direct comparison between theoretical estimates for diffusion-controlled rate constants and our experimental values, the units of k_1 need to be converted to moles of polynucleotide. This is simply done by multiplying k_1 by the polynucleotide chain length, M. The polynucleotides used in the experiments in Figure 2 have an average length, M, of ~ 300 nucleotides for poly(rεA) and ~ 500 nucleotides for poly(dT). If we call k_a the non-co-operative association rate constant for protein binding to the polynucleotide chain (i.e. $k_a = M \times k_1$), then our experimental estimates of k_a are in the range 9×10^8 to $1{\cdot}5 \times 10^9$ M^{-1} (polynucleotide) s^{-1}. These values are to be compared with theoretical estimates from a modified von Smoluchowski (1917) equation for the diffusion-controlled rate constant of a ligand to a polymer chain (Berg *et al.*, 1981):

$$k_a = 4\pi r_g \kappa (D_p + D_D) N_A \, 10^{-3} \left[1 - \frac{\tanh(\eta r_g)}{(\eta r_g)} \right], \tag{8}$$

where r_g is the radius of gyration of the polynucleotide, D_p and D_D are the diffusion coefficients of the protein and polynucleotide, respectively; N_A is Avogadro's number and κ is a steric factor to account for effects associated with proper orientation of the DNA binding site of the protein. Equation (8), without the term in brackets is correct if the polynucleotide were a solid sphere of radius r_g. However, since the polynucleotide is not a solid sphere, the "transparency" term in brackets is necessary. It represents the probability that the protein, upon entering the domain of the polynucleotide, will bind to the polynucleotide rather than pass through it (Berg *et al.*, 1981). From Berg *et al.* (1981) we find:

$$(\eta r_g)^2 = \frac{3L/r_g}{\ln (R_c/b)},$$

where $2L$ is the length of the polynucleotide, b is the radius of the polynucleotide backbone and R_c is the average distance between nucleic acid segments within the domain of one polynucleotide. (R_c is obtained by setting $\pi R_c^2 L = 4/3\pi r_g^3$ and solving for R_c; see Berg & Blomberg (1977) and Berg *et al.* (1981) for details.)

Inserting values of $D_p = 7 \times 10^{-7}$ cm^2 s^{-1} ($D_p \gg D_D$), $r_g = 2 \times 10^{-6}$ to 3×10^{-6} cm (at 0·10 M NaCl; Eisenberg & Felsenfeld, 1967), $R_c = 200$ Å, $b = 10$ Å

and $\kappa = 1/2$, we obtain estimates of $k_a = 2 \times 10^9$ to 3×10^9 M^{-1} (polynucleotide) s^{-1}. Since T4 G32P binds with a definite polarity, with respect to the direction of the nucleic acid sugar-phosphate backbone (Kelly & von Hippel, 1976), this introduces an orientation constraint of at least $\kappa = 1/2$ (it is conceivable that κ may be smaller than 1/2). Our experimental values of $k_a = 9 \times 10^8$ to $1{\cdot}5 \times 10^9$ M^{-1} (polynucleotide) s^{-1} are therefore in good agreement with the approximate theoretical estimates for a diffusion-controlled reaction.

An increase in the molecular weight (length) of the polynucleotide should increase the non-co-operative association rate constant (k_a; in units of M^{-1} (polynucleotide) s^{-1}) since the radius of gyration (i.e. the target size) will increase. We observe this in the measurements with different lengths of poly(rI). k_a does increase from roughly (50) $(6 \times 10^6) = 3 \times 10^8$ M^{-1} (polynucleotide) s^{-1} for the 2·5 S ($\sim$ 50 nucleotide) poly(rI) sample to (1500) $(2 \times 10^6) = 3 \times 10^9$ M^{-1} s^{-1} for the 12 S ($\sim$ 1500 nucleotide) poly(rI) sample. There is insufficient data on radii of gyration of single-stranded homopolynucleotides as a function of molecular weight, under the conditions of our experiments (0·10 M-NaCl) to make quantitative statements. However, an increase of a factor of 10 in k_a certainly seems reasonable for an increase of a factor of 30 in molecular weight.

The salt dependence of k_a(app) in the strong binding region is qualitatively that expected for the diffusion-controlled association between two species of net negative charge. In addition, however, the G32P presumably has a DNA binding site that has a net positive charge. This asymmetric charge distribution on the protein (many DNA binding proteins have isoelectric points < 7) very likely facilitates the correct orientation of the protein near the nucleic acid. Therefore, at very low salt, the approach of the two species (both of net negative charge) is rate-limiting and is facilitated by increasing the salt concentration which screens the electrostatic repulsions. As the salt concentration is increased to $> 0{\cdot}1$ M-NaCl, the Debye screening length drops to < 10 Å. In this salt region the orientation of the DNA binding site of G32P becomes rate-limiting. This orientation step should have a small negative salt dependence (i.e. increased salt concentration will shield the interaction between species of opposite charge and slow the association). The only association that seems to show all aspects of this behavior is the G32P–poly(dT) interaction (see Fig. 4). The salt dependence ($\partial \log k_a / \partial \log [\mathrm{NaCl}]$) is 1·5 in the range 20 mM $<$ [NaCl] $<$ 100 mM, but begins to level off between 0·10 M and 0·30 M-NaCl. Above 0·30 M-NaCl, the pre-equilibrium domain takes over and we observe the salt dependence of the equilibrium constant, K_1. The orientation-limiting region and the pre-equilibrium region discussed above correspond to the screening-controlled and pre-equilibrium regions discussed by Lohman *et al.* (1978).

An alternate interpretation of the association in the strong binding region is that the G32P exists in equilibrium between two conformations, only one of which is capable of binding to the nucleic acid, as shown below:

$$\begin{array}{ccc} & & \overset{k_a}{\rightleftarrows} \\ & \mathrm{P} + \mathrm{D} & \mathrm{PD_{is}}, \\ K_c \uparrow\downarrow & & \underset{k_d}{} \\ & \mathrm{P}^* & \end{array} \qquad (9)$$

where

$$K_c = [P]/[P^*].$$

If the equilibrium between P and P* is rapid, the observed association rate constant would be:

$$k_a(\text{app}) = k_a \frac{K_c}{(1+K_c)}, \tag{10}$$

rather than the true bimolecular rate constant, k_a. If K_c were to decrease with decreasing salt concentration, this might account for the [NaCl] dependence of k_a(app) in the region $< 0{\cdot}10$ M-NaCl. In addition, a rate-limiting step involving a conformational change of the G32P would account for the absence of nucleic acid specificity at low salt.

It is quite possible that something like this is going on at very low salt concentration (e.g. $< 0{\cdot}05$ M-NaCl). However, there are two observations that suggest that an equilibrium involving a G32P conformational change is not important at [NaCl] $\geq 0{\cdot}10$ M. At 0·1 M-NaCl, we observe both pre-equilibrium and strong binding behavior depending on the homopolynucleotide. This argues against a rate-limiting step that involves only the protein. Furthermore, at 0·3 M-NaCl, the G32P–poly(dT) association still displays strong binding behavior with $k_1 = 3 \times 10^6$ M^{-1} (nucleotide) s^{-1}, even though the associations to all other homopolynucleotides are in the weak binding pre-equilibrium limit. Obviously the factors that define the strong binding association behavior are dependent on both the salt concentration and the homopolynucleotide (as well as temperature). This suggests that we need not consider protein conformational changes in our interpretation of the data under strong binding conditions at least in the range $0{\cdot}10$ M $\leq$ NaCl $\leq 0{\cdot}3$ M.

We note at this point that our interpretation of the decrease in k_1 below 0·10 M-NaCl for G32P is different than that for the *lac* repressor–operator association given by Berg *et al.* (1981), Winter *et al.* (1981) and Barkley (1981) in the same [NaCl] region for their experiments with λp*lac*5 (high molecular weight) DNA. However, for the short chain length operator-containing DNA experiments of Winter *et al.* (1981), the qualitative explanations are the same at low salt (i.e. the non-specific (non-co-operative) association becomes rate-limiting and k_1 is measured). Winter *et al.* (1981) observe a salt-independent $k_1 \simeq 2 \times 10^7$ M^{-1} (nucleotide) s^{-1} between 25 mM and 100 mM-NaCl. In our experiments with poly(dT) we observe an essentially salt-independent $k_1 = 3 \times 10^6$ to 4×10^6 M^{-1} (nucleotide) s^{-1} in the range $0{\cdot}10$ M $\leq$ [NaCl] $\leq 0{\cdot}30$ M, but a significant decrease in k_1 below 0·10 M-NaCl (see Fig. 4). As stated above, this decrease may indicate that the approach of the protein and nucleic acid has become rate-limiting, rather than the orientation of the DNA binding site of the protein, or it may reflect a protein conformational change. Goeddel *et al.* (1977) have measured the association of *lac* repressor to 21 and 26 base-pair operator fragments and find that $\partial \log k_a / \partial \log [\text{NaCl}] = -1{\cdot}0$ in the range 50 mM $<$ [NaCl] $< 0{\cdot}20$ M which Lohman *et al.* (1978) have interpreted as indicating that orientation of repressor is rate-limiting for this system.

None of the kinetic observations made in the strong binding region constitutes proof in itself that the strong binding k_a(app) is the bimolecular rate constant for formation of the non-co-operatively bound G32P–nucleic acid complex (k_1 in eqn (11) and Fig. 13). However, collectively they offer a large body of evidence in support of this conclusion. The value of $k_1 = 3 \times 10^6$ to 4×10^6 M^{-1} (nucleotide) s^{-1} compares well with the estimate of $k_1 = 2 \times 10^7$ M^{-1} (nucleotide) s^{-1} by Winter *et al.* (1981) for the non-specific association of *lac* repressor to a 203 base-pair operator-containing fragment. No direct information about dissociation rates can be obtained from the association data presented here since the co-operative interactions are presumably formed before dissociation of the non-co-operatively bound G32P can occur. We have measured the dissociation of co-operatively bound G32P over a wide range of solution conditions and these will be reported elsewhere (Lohman, unpublished results).

(b) *Weak binding, pre-equilibrium association mechanism*

The association kinetics under weak binding conditions display qualitatively different behavior than under strong binding conditions as a function of protein and nucleic acid concentrations, salt, temperature and nucleic acid length. Our observations suggest the following general mechanism for the formation of G32P clusters at low extents of lattice saturation ([NA] $\gg$ [G32P]). The mechanism is also depicted schematically in Figure 13.

$$(1)\quad P + D \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} PD_{is} \qquad \text{(nucleation)} \qquad (11a)$$

$$(2)\quad P + PD_{is} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} PD_{contiguous} \qquad \text{(growth)} \qquad (11b)$$

$$(3)\quad PD_{contiguous} \xrightarrow{k_r} PD_{equilibrium\ cluster\ distribution} \qquad \text{(redistribution)} \qquad (11c)$$

The first step (11a) is the formation of non-co-operatively bound G32P–nucleic acid complexes (PD_{is}), which are in rapid pre-equilibrium with free G32P and nucleic acid binding sites. This step is viewed as a nucleation step since the ensuing co-

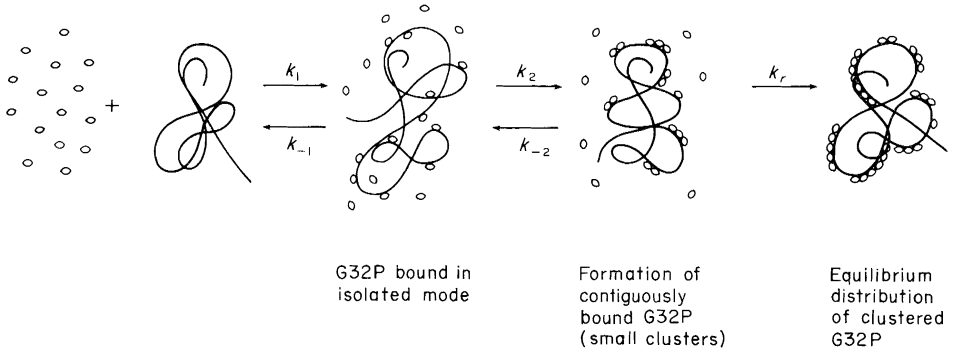

FIG. 13. Schematic of the proposed mechanism for formation of T4 gene *32* protein clusters under conditions of excess nucleic acid. (See also eqns (11).)

operative interaction can only form after some PD_{is} have formed. As we have seen, the concentration of PD_{is} formed in this pre-equilibrium is generally very small (see Fig. 11) since the reactant concentrations are low and K_1 is generally $\leq 10^4$ M^{-1}. In general, this step is missed in the stopped-flow experiment under weak binding conditions since it is fast and has a very small amplitude.

The second step in this mechanism (11b), corresponding to the relaxation time τ_f, represents the growth step, where free G32P contacts the previously established non-co-operatively bound molecules that have provided two singly contiguous sites for formation of co-operative interactions. Upon binding in the proper configuration, a co-operative interaction is formed and the binding constant increases by a factor of ω (10^3 to 10^4). The formation of these co-operative interactions does not eliminate the singly contiguous sites (cluster ends), but rather their concentration should remain fairly constant. At this point we have not specified the mechanism by which the free G32P locates the PD_{is}, or other singly contiguous sites. The possible mechanisms for that step will be discussed in detail in section (e), below. In the scheme of Figure 13 we have drawn the intermediate formed by this second step, with rate constant k_2, as a group of small G32P clusters on the nucleic acid. We simply mean to specify that this second step represents the formation of the first co-operative interactions. This intermediate is rather nebulous and the cluster distribution at this point cannot be well defined. In fact, depending on the conditions, intermediate clusters may be formed that are longer than any that will be found in the final equilibrium cluster distribution as has been observed for the polymerization of actin (Oosawa & Asakura, 1975; Kawamura & Moruyama, 1972).

The third and final step in our proposed mechanism (11c) is the redistribution of G32P to form the final equilibrium distribution of clusters. This step is included because of the observation of the second relaxation time, τ_s (see Fig. 1) under all conditions except very high nucleic acid concentrations. Once the clusters start to form in the second step, they will not immediately establish the most favorable configuration of cluster sizes and some redistribution must occur. This redistribution is likely to be both intramolecular and intermolecular with respect to the nucleic acid. The fact that we observe τ_s indicates that more G32P is binding to the nucleic acid during this redistribution step, otherwise we would not detect a further fluorescence quenching. Since the fluorescent properties of G32P depend only upon whether it is free or nucleic acid bound (see Results), we cannot *directly* detect any interchange of bound G32P among its three different binding modes (isolated, singly or doubly contiguous). However, if the redistribution of G32P on the nucleic acid lattice is slow but results in further binding of free protein, since the free energy of the system will be lowered, then the redistribution process will be observed through the coupled binding of the remaining free protein. No slow relaxation time (τ_s) is observed at very high nucleic acid concentrations, since practically all of the protein is bound before redistribution occurs and hence the redistribution process is invisible.

The presence of the slow relaxation time under conditions of excess nucleic acid is definitely related to the G32P co-operativity. Association experiments with the G32P proteolytic digestion products G32P*I and *III (Moise & Hosoda, 1976) show

this quite nicely. The G32P*I still maintains its co-operativity ($\omega \sim 10^3$; Lonberg *et al.*, 1981) and we observe biphasic association kinetics to single-stranded homopolynucleotides as in the case of G32P. However, the G32P*III, which does not bind co-operatively ($\omega = 1$; Lonberg *et al.*, 1981), displays only a single exponential decay in association experiments with poly(rεA) (Kowalczykowski, unpublished results).

Schwarz (1972) has shown that three relaxation times are expected for the co-operative binding of a ligand to a one-dimensional lattice under conditions of low binding density, high co-operativity and an infinitely long lattice. The fastest relaxation time is associated with the nucleation step, which we do not observe under weak binding conditions. The second relaxation time corresponds to the growth step and is given in equation (12) in our notation (Schwarz, 1972):

$$\tau_2^{-1} = 2k_2[\mathrm{PD_{is}}]. \tag{12}$$

This relaxation time is identical to τ_f^{-1}, as seen in Figure 11, which shows that τ_f^{-1} is directly proportional to $[\mathrm{PD_{is}}]$. The third relaxation time, as discussed by Schwarz (1972), is due to redistribution of bound ligands. We have suggested above that the slow relaxation time that we observe (τ_s) may represent a redistribution of bound G32P to form the most favorable cluster distribution. At this point, however, we cannot make a quantitative statement about the rate-limiting step in this redistribution process.

(c) *Primary factors that determine the rate-limiting step in the association (i.e. strong binding* versus *weak binding behavior)*

On the basis of the proposed mechanism for the formation of co-operatively bound G32P–polynucleotide complexes, we can ask what causes the differences between the strong binding and weak binding association behavior. Primarily, what is the basis for the salt concentration effect that shifts the rate-limiting step in the association so that one measures k_1 under strong binding conditions, but observes a pre-equilibrium under weak binding conditions? This is explained by our proposed scheme (see equations (11) and Fig. 13), if the following conditions are met:

$$2k_2[\mathrm{PD_{is}}] > k_{-1} \qquad \text{strong binding (measure } k_1) \tag{13}$$

$$k_{-1} > 2k_2[\mathrm{PD_{is}}] \qquad \text{weak binding (pre-equilibrium).} \tag{14}$$

Under weak binding conditions $k_{-1} > 2k_2[\mathrm{PD_{is}}]$ and the first step quickly establishes a pre-equilibrium situation, while the growth step with rate constant k_2 is rate-limiting. However, as previously shown (see Fig. 10) the non-co-operative binding constant for the G32P–polynucleotide interaction is extremely salt-dependent. In addition the majority of this salt dependence is contained in the dissociation rate constant (Lohman *et al.*, 1978; Lohman, 1980 and unpublished results; Kowalczykowski *et al.*, 1980). As a result, upon lowering the [NaCl], k_{-1} decreases dramatically. At a low enough [NaCl], the relative magnitudes of k_{-1} and $k_2[\mathrm{PD_{is}}]$ change so that $2k_2[\mathrm{PD_{is}}] > k_{-1}$. Under these conditions, the rate-limiting step in the association becomes k_1.

We can obtain a reasonable estimate of the value of k_{-1} at which the crossover from strong binding to weak binding behavior occurs. From equations (5), (13) and (14) we see that the value of k_{-1}, relative to τ_f^{-1}, is the determining factor. From Figures 5, 6, 7 and 8 we see that τ_f^{-1} does not usually exceed $80\ s^{-1}$ under weak binding conditions, where k_{-1} must be greater than τ_f^{-1}. Furthermore, strong binding behavior will be observed when $\tau_f^{-1} > k_{-1} \simeq 10\ s^{-1}$. Therefore the crossover should occur within the range $10\ s^{-1} < k_{-1} < 80\ s^{-1}$ or equivalently, $4 \times 10^4\ M^{-1} < K_1 < 3 \times 10^5\ M^{-1}$, since k_1 should be fairly constant above 0·10 M-NaCl. As a result, any set of conditions (mainly [NaCl], temperature and nucleic acid lattice) that yields a value of $k_{-1} < 10$–$80\ s^{-1}$ will result in the measurement of k_1 in an association experiment. This explains the fact that the [NaCl] range over which strong binding association behavior is observed is dependent on the particular homopolynucleotide. We have shown (Lohman, 1980, and unpublished results) that essentially all of the base and sugar specificity that has been observed for the equilibrium constant, K (Newport *et al.*, 1981) originates in the dissociation rate constant. Since k_{-1} for the G32P–poly(dT) dissociation is lower than for any other polynucleotide, strong binding behavior persists to a much higher [NaCl] ($\sim$ 0·3 M) than for any of the other polynucleotides (i.e. one needs to raise the [NaCl] to $>$ 0·30 M in order to increase k_{-1} so that it is $>$ 10 to $80\ s^{-1}$). In addition, the crossover from strong binding to weak binding behavior is observed to occur over a narrow [NaCl] range in Figure 4. This is presumably because as the [NaCl] is raised, k_{-1} increases dramatically and $k_2[PD_{is}]$ (or τ_f^{-1}) decreases. Either of these changes would result in a switch in rate-limiting steps; however, the two acting in concert will cause the shift to occur quite abruptly, as a function of [NaCl].

(d) [*NaCl*] *dependence of the strong binding (diffusion-controlled)* versus *weak binding (pre-equilibrium) associations*

As Lohman *et al.* (1978) have shown, the salt dependence of the observed association rate constant is sensitive to whether an association reaction occurs *via* a pre-equilibrium mechanism or is diffusion-controlled. For a diffusion-controlled association, $|\partial \log k_a/\partial \log [NaCl]| \ll |\partial \log K/\partial \log [NaCl]|$. As discussed above, the basis for this is that the effect of salt concentration on k_a will be only to screen the reacting species and hence will generally be small (Lohman *et al.*, 1978).

For an association that occurs *via* a pre-equilibrium formation of a protein–nucleic acid complex, we expect $|\partial \log k_a(app)/\partial \log [NaCl]| \simeq |\partial \log K/\partial \log [NaCl]|$, since $k_a(app)$ possesses the entire salt dependence of the equilibrium constant, K (Lohman *et al.*, 1978). For most DNA binding proteins, K decreases with increasing [NaCl] due to the direct release of counterions from the nucleic acid and/or protein upon formation of ionic interactions in the complex (Record *et al.*, 1976,1978; not simply a screening effect as in the diffusion-controlled case). That is to say, the actual reaction for the pre-equilibrium step should be written as:

$$P + D \rightleftarrows PD_{is} + xNa^+ + yCl^- \tag{15}$$

(both cations and anions are released in the case of G32P (Kowalczykowski *et al.*, 1981*b*)). An increase in [NaCl] will decrease the number of nucleation sites, PD_{is},

and hence k_a(app), under weak binding conditions, by shifting the equilibrium in (15) to the left (Lohman *et al.*, 1978; Record *et al.*, 1976,1978). As we have shown, a pre-equilibrium exists under weak binding conditions and $(\partial \log k_a(\text{app})/\partial \log [\text{NaCl}]) = -5 \pm 1$ for poly(rεA) and poly(rI) and $-3{\cdot}6$ for poly(dT) (see Fig. 4). These values agree very well with those found for $\partial \log K/\partial \log [\text{NaCl}]$ (Kowalczykowski *et al.*, 1981*b*; Newport *et al.*, 1981), which indicates that the association involves a pre-equilibrium (Lohman *et al.*, 1978). Hence, the salt dependence of k_a(app) is a helpful diagnostic to determine if a pre-equilibrium mechanism is in operation in a particular protein–nucleic acid association.

(e) *The mechanism of the growth step in the pre-equilibrium association*

As one can see from the scheme in Figure 13, the second step in the proposed mechanism (growth of clusters) has not been specified. In this section we discuss the various pathways by which a G32P, free in solution, may find its way to a non-co-operatively bound G32P in order to form the first co-operative interaction and start the growth of G32P clusters. Once the pre-equilibrium formation of non-co-operatively bound G32P has occurred in the first step, the second step appears quite analogous to the well studied association of the *E. coli lac* repressor to its operator, which is contained in a large DNA molecule possessing many non-specific binding sites (Riggs *et al.*, 1970). In the G32P case, however, the specific sites of interaction are the singly contiguous sites on either side of a non-co-operatively bound G32P molecule rather than a specific nucleotide sequence. When a G32P molecule reaches a singly contiguous site, its binding constant increases by a factor of $\omega(\sim 10^3$ to $10^4)$. Under physiological conditions, the increase in binding constant when *lac* repressor moves from a non-specific site to the operator is $\sim 10^8$ to 10^9 (von Hippel *et al.*, 1974,1975; Lin & Riggs, 1975; Record *et al.*, 1977). In the case of repressor this specificity is salt-dependent, since the non-specific and specific binding constants have different salt dependences, whereas for G32P ω seems to be independent of [NaCl]. Hence the *lac* repressor–operator association and the growth step of the G32P–polynucleotide association are quite similar and we can draw on the detailed theoretical literature that has developed for the repressor–operator kinetics (Richter & Eigen, 1974; Berg & Blomberg, 1976,1977,1978; Schranner & Richter, 1978; Berg *et al.*, 1981) to interpret our experimental values of k_2 in terms of a specific mechanism. There are basically three pathways by which free G32P can reach a singly contiguous site on the single-stranded polynucleotide (Berg & Blomberg, 1976,1978; Berg *et al.*, 1981).

(1) Three-dimensional diffusion of the G32P directly to the singly contiguous site.

(2) Hopping, whereby the G32P first binds non-co-operatively (non-specifically) and then undergoes a series of microscopic dissociations and reassociations until it contacts a singly contiguous site.

(3) Sliding, whereby the G32P also binds non-co-operatively and then slides (i.e. undergoes a random one-dimensional diffusion along the chain without dissociation) with intermittent dissociations and reassociations, until it contacts a singly contiguous site.

In pathways (2) and (3), the translocation step (either hopping or sliding) is rate-limiting at high salt. Pathways (2) and (3) may seem identical or the differences may appear inconsequential; however, as pointed out by Berg *et al.* (1981), there are important differences that can be sorted experimentally. The sliding and hopping pathways both utilize the fact that the non-specific binding sites are correlated due to their physical linkage in a linear lattice. In this sense, the association of the G32P to a singly contiguous site is facilitated by the simple fact that the singly contiguous site is attached to the rest of the nucleic acid. This general acceleration of an association reaction due to a reduction in dimensionality was originally proposed by Adam & Delbrück (1968). The difference between hopping and sliding is the mechanism by which these non-specific sites are sampled by the protein. Hopping, by definition, is a mechanism that is utilized by all DNA binding proteins since it involves a series of *microscopic* dissociations and reassociations to strongly correlated regions of the lattice (Berg & Blomberg 1976; Berg *et al.*, 1981). Microscopic dissociations can be thought of as those that carry the protein sufficiently away from the lattice so that the counterions can recondense (Manning, 1969,1978; Record *et al.*, 1976,1978) but not far enough away so that it loses correlation with the site from which it dissociated (Berg *et al.*, 1981). Upon reassociating, the protein has some probability distribution for hitting a number of sites within the immediate vicinity of the site that it left. A series of these hops allows the protein to sample many binding sites. Sliding is the random, one-dimensional translocation of the protein without dissociating (i.e. the protein still maintains some of its contacts with the polynucleotide lattice). As Berg *et al.* (1981) discuss, hopping is not facilitating in the sense that it occurs for all proteins. However, in the absence of a facilitating mechanism such as sliding, hopping does enable the protein to accelerate its association to a specific site on the DNA (compared with three-dimensional diffusion) *via* use of the non-specific DNA. However, this is an accelerated rate compared to the standard mathematical estimate of a diffusion-controlled rate, which does not normally include a mechanism such as hopping, since the calculation is usually done for independent binding sites. It is only necessary to consider hopping when the binding sites are linked as in a polynucleotide. Sliding is quite probably a facilitating mechanism, since we expect that not all proteins are capable of sliding along the lattice. (The answer to this must await experiments on a number of protein–nucleic acid systems.)

In the case of the *lac* repressor–operator association, there is good evidence as judged by the excellent fit of the experimental data (Riggs *et al.*, 1970; Barkley 1981; Winter *et al.*, 1981) to Berg and Blomberg's theory, that repressor does slide along double-stranded DNA with a sliding rate constant, $k_s \sim 10^6\ s^{-1}$. We wish to compare our experimental values of k_2 to those predicted by Berg & Blomberg's (1976,1977,1978) theory for the three pathways listed above. This will lend some insight into the most likely mechanism for the growth step. The experimental values of k_2 are obtained from the slopes of the plots of τ_f^{-1} *versus* $[PD_{is}]$ (see equation (5) and Fig. 11) and are listed in Table 3. Alternatively, k_2 can be obtained from the plateau value, $\tau_f^{-1}(\infty)$, since $\tau_f^{-1}(\infty) = 2k_2[G32P]_{total}$. With one exception (poly(rA), 0·10 M-NaCl), the experimental values of k_2 are all in

excess of 10^8 M^{-1} (contiguous site) s^{-1} and decrease slightly with increasing salt concentration.

The theoretical estimates of k_2 are obtained from the work of Berg & Blomberg (1976,1977,1978) and Berg *et al.* (1981) for the association of a protein to N specific sites (the N singly contiguous sites in the case of G32P), which are part of a large nucleic acid lattice. Since our experimental estimates of k_2 have been determined on a per (singly contiguous) site basis, the theoretical equations for k_2, which are given below, have N set equal to one. For the case of three-dimensional diffusion directly to a singly contiguous site on a polynucleotide (using our notation):

$$k_2 = k_1/(1 + D_T K_1), \tag{16}$$

where k_1 is the non-co-operative association rate constant in units of M^{-1} (nucleotide) s^{-1} (not M^{-1} (polynucleotide) s^{-1}), D_T is the concentration of nucleotides and K_1 is the non-co-operative equilibrium constant. The factor $(1 + D_T K_1)$ accounts for the fact that protein can be "trapped" at isolated sites if non-co-operative binding is tight.

For the two-step process of non-co-operative binding followed by sliding of the G32P (without hopping) until a singly contiguous site is contacted (Berg & Blomberg, 1976,1978; Berg *et al.*, 1981):

$$k_2 = \frac{k_1 (k_s/k_{-1})^{\frac{1}{2}}}{(1 + D_T K_1)}, \tag{17}$$

where k_s is the sliding rate constant and k_{-1} is the rate constant for dissociation of non-co-operatively bound G32P. Equation (17) is the expression for k_2 in the high salt limit of weak non-co-operative binding, which applies to the conditions of our experiments. It does not include the coupling of intermittent dissociations of the protein during the sliding process, although Berg & Blomberg (1976,1978) have developed a more general expression which must be used when $K_1 D_T \geq 1$. The term $(k_s/k_{-1})^{\frac{1}{2}}$ in equation (17) is the length (in nucleotides) that the target site is "extended" due to the ability of the protein to slide along the nucleic acid. That is to say, if the G32P binds within $(k_s/k_{-1})^{\frac{1}{2}}$ nucleotides of a singly contiguous site, it will contact that site during its lifetime as a non-co-operatively bound species.

The expression for k_2 for the case of non-co-operative binding followed by hopping of G32P until a singly contiguous site is contacted is (Berg *et al.*, 1981):

$$k_2 = \frac{1}{2} \frac{1{\cdot}5 \pi b D_p N_A 10^{-3}}{(1 + D_T K_1)}, \tag{18}$$

where D_p ($\simeq 7 \times 10^{-7}$ cm^2/s) is the three-dimensional diffusion coefficient of the G32P and b is a reaction radius for the interaction with a nucleotide (b is taken to be ~ 15 Å).

As a comparison calculation we have chosen the G32P association to poly(rεA) at 0·25 M-NaCl, 25·0°C; [G32P] = $2{\cdot}1 \times 10^{-7}$ M and [poly(rεA)] = $1{\cdot}0 \times 10^{-5}$ M (nucleotides). Under these conditions the experimental value of k_2 is $2{\cdot}5 \times 10^8$ M^{-1} (contiguous site) s^{-1}. From the known values of $k_1 = 3 \times 10^6$ M^{-1} (nucleotide) s^{-1} and $K_1 = 8 \times 10^3$ M^{-1} we obtain $k_{-1} = k_1/K_1 = 330$ s^{-1}. Using these we calculate the following estimates for k_2.

(1) Direct three-dimensional diffusion (see equation (16)):

$$k_2 \simeq 3 \times 10^6 \text{ M}^{-1} \text{ (contiguous site) s}^{-1}.$$

(2) Non-co-operative binding, sliding (see equation (17)):

$$k_2 = 2{\cdot}5 \times 10^8 \text{ M}^{-1} \text{ (contiguous site) s}^{-1} \text{ (using } k_s = 2{\cdot}3 \times 10^6 \text{ s}^{-1}).$$

(3) Non-co-operative binding, hopping (see equation (18)):

$$k_2 \simeq 1{\cdot}3 \times 10^8 \text{ M}^{-1} \text{ (contiguous site) s}^{-1}.$$

The theoretical estimate of k_2 with sliding has been made to agree with the experimental value by using the sliding rate constant, k_s, as an adjustable parameter.

The above calculations indicate that three-dimensional diffusion of G32P directly to the singly contiguous site is eliminated as a possibility. However, both calculations which consider hopping and sliding are consistent with the experimental value of k_2. Hence we conclude that, in the growth step, free G32P makes use of its affinity for the isolated nucleic acid sites and translocates (by hopping and/or sliding) until it contacts the singly contiguous binding sites. As Berg *et al.* (1981) discuss, the estimate of k_2 with hopping (equation (16)) has been derived under idealized conditions which assume, among other things, that k_1 is diffusion-controlled. As a result, the numerical value of k_2 obtained from equation (16) is not very useful as a direct comparison to experimental values since we do not know whether all of the assumptions pertain to gene *32* protein. This is not to say that hopping may not be important; however, it is unlikely to be as efficient a mechanism for a protein to sample nucleic acid sites as sliding. The sliding calculation is certainly more rigorous and a direct comparison to experimental results is meaningful.

An interesting outcome of the sliding calculation is that the value of $k_s = 2 \times 10^6 \text{ s}^{-1}$ obtained for G32P is very close to the value of $k_s \simeq 10^6 \text{ s}^{-1}$ which is found to fit the *lac* repressor–operator association data (Berg & Blomberg, 1976,1978; Barkley, 1981; Winter *et al.*, 1981). (Note that $k_s \equiv D_1/l^2$ where l is the length of a base-pair ($3{\cdot}4 \times 10^{-8}$ cm) and D_1 is the one-dimensional diffusion coefficient used in previous calculations by Berg & Blomberg (1976,1978). Therefore $k_s = 2{\cdot}3 \times 10^6 \text{ s}^{-1}$ corresponds to $D_1 = 2{\cdot}7 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$.) Similar calculations using equation (17), yield estimates of k_s in the range 7×10^5 to $3 \times 10^6 \text{ s}^{-1}$ from experiments with the other homopolynucleotides listed in Table 3 and hence k_s seems to be independent of base composition. This range of values for k_s is quite reasonable by comparison with the *lac* repressor result.

In principle, a valuable diagnostic for a sliding mechanism is the [NaCl] dependence of k_2. From equation (17), we can write the expected salt dependence of k_2 in the range of [NaCl] > 0·10 M as:

$$\frac{\partial \log k_2}{\partial \log [\text{NaCl}]} = \frac{\partial \log k_1}{\partial \log [\text{NaCl}]} + \frac{1}{2} \frac{\partial \log k_s}{\partial \log [\text{NaCl}]} - \frac{1}{2} \frac{\partial \log k_{-1}}{\partial \log [\text{NaCl}]} \simeq -3. \qquad (19)$$

This is one-half the salt dependence of the equilibrium constant, K_1. In arriving at the predicted salt dependence in equation (19) we have assumed that the sliding rate constant, k_s, is independent of salt concentration since this seems to be the case for the interaction of *lac* repressor with double-stranded DNA (Berg & Blomberg, 1978; Barkley, 1981; Winter *et al.*, 1981). In addition, k_1 should be essentially independent of [NaCl] in the region of [NaCl] > 0·10 M. This is observed in the poly(dT) association where we can measure k_1 up to 0·30 M-NaCl (see Fig. 4). Although $(\partial \log k_1/\partial \log [\text{NaCl}]) = 1{\cdot}5$ below 0·10 M-NaCl, we observe that k_1 is fairly insensitive to [NaCl] in the range 0·10 M < [NaCl] < 0·30 M. We cannot measure k_1 above 0·30 M-NaCl, although its salt insensitivity is expected to continue to higher [NaCl]. As a result, k_{-1} will possess the [NaCl] dependence of K_1^{-1} (Lohman *et al.*, 1978; Berg & Blomberg, 1978; Lohman, unpublished results).

In the case of gene *32* protein we observe $(\partial \log k_2/\partial \log [\text{NaCl}]) = -0{\cdot}9 \pm 0{\cdot}3$ (see Table 3) for poly(rεA) in the range 0·20 M ≤ [NaCl] ≤ 0·40 M and for poly(dT) in the range 0·46 M ≤ [NaCl] ≤ 0·90 M. The observed salt dependence is only one-third of the predicted value. One possible explanation is that the assumption made in equation (19), that k_s is independent of [NaCl], may be incorrect for gene *32* protein. An additional possibility is that our experimental determinations of k_2 are in a [NaCl] region where sliding becomes less efficient and that a combination of hopping and sliding is occurring. In fact, this is reasonable since we have evidence of a switch in the mechanism for dissociation of G32P from the ends of protein clusters as a function of [NaCl] (Lohman, unpublished results). Since the hopping estimate of k_2 is independent of [NaCl] (see equation (18)), a combination of hopping and sliding would produce a [NaCl] dependence in the range that we observe. A further possibility is that we have artificially constrained $\tau_f^{-1}(\infty)$ and hence k_2 (since $\tau_f^{-1}(\infty) = 2k_2P_{tot}$) to be salt-independent through use of the low salt value of $\tau_f^{-1}(\infty)$ to analyze the associations at higher salt. Recall that this is done since accurate values of $\tau_f^{-1}(\infty)$ are difficult to obtain at high salt since K_1 is so small. As a result, we are left with some uncertainty in the salt dependence of k_2, and hence we cannot draw any firm conclusions about a mechanism from it.

The experimental estimates of k_2 in Table 3 seem to be fairly independent of homopolynucleotide; however, the NaCl concentration range in which they were obtained does vary with the nucleic acid lattice. When the values of k_2 are extrapolated to a common [NaCl], nucleic acid specificity becomes apparent in the order of increasing k_2; poly(rA) < poly(rU) < poly(rεA) ~ poly(rI) < poly(dU) < poly(dT). This qualitative result is predicted by the sliding model (see equation (17)) since $k_2 \propto (k_{-1})^{-\frac{1}{2}}$ and at a constant [NaCl], k_{-1} decreases in going from poly(rA) to poly(dT), following the hierarchy listed above (Lohman, unpublished results). However, since the experimental [NaCl] dependence of k_2 is not firm, as discussed above, this extrapolation and its conclusion are questionable.

We have also obtained data on the temperature dependence of k_2, which is shown in Table 3, for the G32P–poly(rεA) association at 0·1 M-NaCl for $T \leq 20{\cdot}0$°C (at $T \geq 25{\cdot}0$°C, the association reaction measures only k_1). The data yield much higher values of k_2 than those obtained at higher temperatures (25·0°C) and higher [NaCl]. We observe that k_2 is highly temperature-dependent; it increases from $3{\cdot}5 \times 10^8$ M^{-1} (contiguous site) s^{-1} at 10·0°C to $3{\cdot}0 \times 10^9$ at 20·0°C, with an apparent

activation energy of 35 kcal/mol. An extrapolation to 25·0°C yields a value of $k_2 \sim 7 \times 10^9$ M^{-1} (contiguous site) s^{-1}, which is much larger than the values measured at 0·10 M-NaCl, 25·0°C for poly(rA) or poly(rU) or for the other homopolynucleotides at 25·0°C, but higher [NaCl]. This apparent increase in k_2 at 0·10 M-NaCl might reflect an increased efficiency in the sliding mechanism (since k_{-1} decreases), so that it dominates over hopping at low salt. However, since the experimental values of K_1, which were used to calculate [PD_{is}] and hence k_2, are anomalously low (see Results section (b) (vii), and Table 4) we cannot place much emphasis on these temperature studies.

On the basis of the calculations presented in this section, our main conclusion is that gene *32* protein is able to translocate along single-stranded nucleic acids and that it does use the isolated regions of the polynucleotide to accelerate the formation of contiguously bound protein clusters. We cannot rigorously determine whether gene *32* protein actually slides as *lac* repressor seems to do. In fact, there is a significant difference between the repressor–operator association and the G32P system discussed here. Since G32P can translocate, the singly contiguous sites that are set up in the pre-equilibrium are mobile. This may slightly alter the calculations, since this mobility of the sites should be reflected in k_2.

5. Conclusions and implications for the role of gene *32* protein during replication

We have proposed a mechanism for the non-co-operative association of G32P to single-stranded polynucleotides and the subsequent formation of co-operatively bound G32P clusters, under conditions of excess nucleic acid (see equations (11) and Fig. 13). By varying the solution conditions (mainly [NaCl]), we have been able to measure the non-co-operative association rate constant, k_1, the non-co-operative equilibrium constant, K_1 (and hence k_{-1}) and k_2, the rate constant for the binding of G32P to a singly contiguous site (growth step). This represents the first measurement of the non-specific association rate constant (k_1) for a protein–nucleic acid interaction. Furthermore, through comparisons of our experimental values of k_2 for the growth step with theoretical estimates of k_2 (Berg & Blomberg, 1976,1978; Berg *et al.*, 1981) we conclude that G32P does translocate along single-stranded nucleic acids. In light of the evidence found for *lac* repressor (Richter & Eigen, 1974; Berg & Blomberg, 1976,1978; Barkley, 1981; Winter *et al.*, 1981), oligolysines (Pörschke, 1979*a*) and T4 gene *32* protein, the ability to translocate along nucleic acids may be a fairly general property of DNA binding proteins. Certainly there are many cases of enzymes that must slide, even though sliding has never been directly measured, simply by the fact that these enzymes carry out their functions processively. For example, enzymes such as RNA polymerase, DNA polymerase, helicases (Scott *et al.*, 1977; Yarranton *et al.*, 1979*a*,*b*) and the T4 phage u.v. repair enzyme (Lloyd *et al.*, 1980) must slide in order to function in a processive manner.

What can we infer about the *in vivo* action of gene *32* protein from the finding that it does translocate along single-stranded nucleic acids? The role of G32P during recombination and repair as well as the autogenous regulation of its

synthesis is quite likely to be passive in the sense that G32P is probably only needed to saturate the transiently formed regions of single-stranded DNA or RNA. Whether this is also true for its role in DNA replication is not a settled question. Certainly parts of its function during replication are passive as described above; however, Alberts *et al.* (1980) have evidence suggesting that G32P may play an active role in opening the replication fork while it is associated with DNA polymerase and the accessory proteins. The ability of G32P to translocate may enable it to move along with DNA polymerase during leading strand synthesis. Translocation would also be extremely useful for its passive role of binding co-operatively to and saturating a newly formed stretch of single-stranded DNA or RNA. Since these transient single-stranded regions are formed rapidly, G32P must possess a reasonably fast and efficient means for attaining saturation. Even though the protein binds co-operatively at equilibrium it still must contact a singly contiguous site on the lattice in order for the co-operativity to be realized. Certainly, if the G32P can translocate along the lattice it will be able to sample sites and locate singly contiguous binding sites more rapidly than by successive macroscopic dissociations and reassociations. This would also accelerate the rate at which the G32P can saturate its own messenger RNA in order to control its synthesis (Gold *et al.*, 1976; Russel *et al.*, 1976; Lemaire *et al.*, 1978). Whether or not translocation of G32P is beneficial during replication for anything other than accelerating saturation of the transient single strands must await more detailed knowledge of the replication fork and its configuration with respect to the proteins involved in replication.

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